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14. ABSTRACT There is high comorbidity between post-traumatic stress disorder (PTSD) and alcohol dependence, indicating that exposure to stressful events increases the risk of alcoholism. Thus, identifying pharmacological targets with potential therapeutic value in treating PTSD-associated alcoholism is critical. An interesting candidate is neuropeptide Y (NPY). Recent evidence suggests that low NPY levels promote high alcohol consumption, and it has been established the NPY protects against stress and anxiety. The overall goal of this grant is to determine the role of NPY (and related neuropeptides) in modulating stress-induced increases of alcohol consumption using mouse models. The specific projects for the current funding year determined if A) overexpression of brain NPY with a recombinant adeno-associated virus (rAAV) vector is protective against increased alcohol consumption, and B) if mutant mice lacking normal production of NPY show enhanced sensitivity to stress-induced increases of ethanol consumption. Results indicate that overexpression of brain NPY protects against high alcohol drinking in mice, and that a lack of NPY in mutant mice increases sensitivity to stress-induced alcohol self-administration. Together, the current findings provide evidence that NPY signaling protects against the effects of stress on excessive alcohol self-administration. Thus, NPY may have therapeutic value in treating alcoholism triggered by PTSD.

15. SUBJECT TERMS
Alcohol, Alcoholism, Ethanol, Post-Traumatic Stress Disorder, Mice, Neuropeptide Y, Relapse

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INTRODUCTION: People who have been exposed to an extremely traumatic event, such as witnessing a death, receiving a threat of death, or experiencing a serious injury, may develop a set of symptoms known as posttraumatic stress disorder (PTSD). Events that contribute to the development of PTSD are common to individuals placed in a combat environment. Evidence suggests that there is a high comorbidity between PTSD and alcohol dependence. Given the prevalence of PTSD among veterans of war and the increased risk of alcoholism for individuals suffering from PTSD, identifying pharmacological targets with potential therapeutic value in treating PTSD-associated alcoholism may be considered of high relevance to the U.S. military. An interesting candidate is neuropeptide Y (NPY), and neurochemical that is present throughout the central nervous system. NPY is involved with a diverse set of biological functions including the integration of emotional behavior such as anxiety and depression. Interestingly, recent evidence suggests that low NPY levels and deletion of NPY or the NPY Y1 receptor promote high alcohol consumption. Furthermore, combat-related PTSD is associated with decreased plasma levels of NPY, and uncontrolled stress caused by exposure to military survival training results in depletion of plasma NPY levels following extended exposure. Because low NPY levels promote increased alcohol intake, reduced NPY associated with PTSD may be a factor that leaves individuals susceptible to alcoholism. **Therefore, the guiding hypothesis of the present proposal is that normal NPY signaling protects against uncontrolled alcohol drinking and relapse caused by exposure to stressful events.** To address this issue, a set of studies have been proposed using animal models of stress-induced alcohol consumption. Mutant mice lacking normal NPY signaling, and overexpression of NPY with the use of a recombinant adeno-associated virus (rAAV) vector that causes expression and constitutive secretion of NPY (rAAV-FIB-NPY), are powerful tools used in the present research. These studies will establish if normal NPY signaling protects against the effects of stress on uncontrolled alcohol drinking and relapse of alcohol-seeking behavior.

BODY: The experiments described below fall into 2 categories: Those related to Tasks 1 and 3 of the Statement of Work, and additional experiments that were run which complement work that is outlined in this proposal. We completed Task 1, and performed experiments that provided additional insight into questions associated with Task 3 (a task that we completed in the previous funding year).

TASK 1: Determine if mutant mice lacking production of NPY show enhanced sensitivity to uncontrolled alcohol self-administration caused by exposure to foot-shock stress.

Drinking in the dark (DID) procedures have recently been developed to induce excessive ethanol drinking in C57BL/6J mice which result in blood ethanol concentrations reaching levels that have measurable affects on physiology and/or behavior. We used this procedure to assess the effects of uncontrolled and excessive ethanol drinking in NPY-/- and NPY+/+ mice during exposure to psychological stress. Repeated intraperitoneal injections of saline has been reported to cause stress-induced increases of ethanol drinking by mice, and we used this procedure (rather than shock) to determine the effects of stress in our mice. Mice received access to ethanol (20% v/v) in place of water for 2-hours beginning 3-hours into the dark cycle (the DID procedure). In Experiment 1, mice were given an intraperitoneal injection of saline 30-minutes before receiving their ethanol bottle. Experiment 2 was similar except that mice were not given the saline injection stressor before each day of DID procedures. **Figure 1** shows data from Experiment 1. Relative to NPY+/+ mice, NPY-/- mice showed significantly higher levels of ethanol consumption over the 3-days of the experiment. On the other hand, there were no significant differences between NPY-/- and NPY+/+ mice in ethanol consumption when no saline injection were given (**Figure 2**). These data show that NPY-/- mice are more sensitive the

effects of stress (caused by intraperitoneal injections) on ethanol consumption. Thus, NPY is protective against excessive alcohol drinking stemming from stress exposure, consistent with our guiding hypothesis. This work is currently in preparation for publication.

TASK 3: Determine if transduction of a NPY viral vector (rAAV-FIB-NPY) into the amygdala of C57BL/6J mice protects against uncontrolled alcohol self-administration caused by exposure to stress.

In the previous funding years we showed the overexpression of NPY in the amygdala with a rAAV-FIB-NPY vector protected against excessive ethanol drinking associated with the stress of ethanol deprivation in C57BL/6J mice and in rats selectively bred for high ethanol drinking. Our collaborator recently developed a rAAV-FIB-NPY-13-36 viral vector, which causes overexpression and secretion of the NPY-13-36 peptide fragment in the amygdala. Importantly, the NPY-13-36 fragment is selective to the NPY Y2 receptor, and thus we can determine the role of amygdalar NPY Y2 receptors in excessive ethanol drinking with this tool. We gave bilateral injections of this vector into the amygdala of C57BL/6J mice. Mice were then trained to press a lever to gain access to ethanol reinforcement on one lever and water reinforcement on a second lever. Following training, mice were given limited access to ethanol in 2 hour daily sessions. Consistent with our previous observations with the rAAV-FIB-NPY vector, the rAAV-FIB-NPY-13-36 vector significantly reduced preference for ethanol reinforcement relative to the rAAV-FIB-GFP control treatment (**Figure 3**; preference ratio = lever presses for ethanol / total lever presses). These observations replicate our previous work by showing that NPY signaling in the amygdala modulates ethanol intake, and extend these findings by showing that the NPY Y2 receptor is involved. This work is currently in preparation for publication.

ADDITIONAL RELATED RESEARCH: NPY signaling in the nucleus accumbens modulates ethanol-induced locomotor sensitization. Numerous studies have demonstrated the ability of repeated ethanol injections to induce a persistent condition in which mice show enhanced susceptibility to the locomotor-stimulating effects of ethanol, a phenomenon labeled behavioral sensitization. We used the rAAV-FIB-NPY-13-36 viral vector and the NPY-/- mice to show that NPY signaling in the core region of the nucleus accumbens modulates ethanol-induced behavioral sensitization. Mice were habituated to a locomotor chamber and intraperitoneal (i.p.) saline injection over 3-days. On day 4, mice received an i.p. injection of a 1.5 g/kg dose of ethanol before placement into the locomotor chamber (initial ethanol). For the following ten days, mice received a 2.5 g/kg i.p. ethanol injection in their home cage. On test day, all mice were given an i.p. injection of a 1.5 g/kg dose of ethanol (test day) and a saline injection on the last day of the experiment (final saline). As can be seen in **Figure 4**, DBA/2J mice showed increased ethanol-induced locomotor activity following repeated homecage ethanol injections (initial ethanol versus test day). Importantly, mice given bilateral injection of the rAAV-FIB-NPY-13-36 vector into the nucleus accumbens core showed blunted ethanol-induced behavioral sensitization relative to mice treated with the rAAV-FIB-GFP control vector, indicating that Y2 receptor signaling in this regions modulates sensitization. Since the Y2 receptor is presynaptic and blunts endogenous NPY release, these observations suggest that endogenous NPY signaling positively modulates ethanol-induced locomotor sensitization. Consistent with this view, mutant mice lacking NPY (NPY-/- mice) failed to show ethanol-induced behavioral sensitization after repeated ethanol injections, an effect evident in normal NPY+/+ mice (**Figure 5**). Since behavioral sensitization is thought to reflect sensitivity to the rewarding properties of ethanol, the observations provide novel evidence that NPY signaling modulates ethanol reinforcement. This work is currently in preparation for publication.

KEY RESEARCH ACCOMPLISHMENTS: A list of key research accomplishments achieved during the third budget year of this grant are as follows:

- Establishing that NPY signaling is protective against the effects of stress on excessive ethanol drinking, as NPY-/- mice show greater ethanol drinking after the stress of intraperitoneal injections relative to NPY+/+ mice.

- Establishing that overexpression of a NPY Y2 receptor agonist (via the rAAV-FIB-NPY-13-36 vector) in the amygdala protects against excessive alcohol drinking in C57BL/6J mice. Thus, the Y2 receptor in the amygdala modulates ethanol intake.

- Establishing that overexpression of the NPY Y2 receptor agonist (via the rAAV-FIB-NPY-13-36 vector) in the core region of the nucleus accumbens blunts ethanol-induced behavioral sensitization, evidence that Y2 receptor signaling in this regions modulates sensitization.

- Establishing that normal NPY expression is required for ethanol-induced behavioral sensitization, as NPY-/- mice fail to show this phenotype.

REPORTABLE OUTCOMES: The following is a list publications and published abstracts that have been supported by this grant during the third budget year:

PUBLICATONS

1. Cubero, I., Carvajal, F., de la Torre, L., Navarro, M., Sanchez-Amate, C., & Thiele, T. E. (under review). MC4-R signaling in the nucleus accumbens shell, but not in the paraventricular hypothalamus, modulates ethanol self-administration in Sprague-Dawley rats. *Alcoholism: Clinical & Experimental Research.*

2. Lowery, E. G. & Thiele, T. E. (under review). Animal models for stress-induced alterations of ethanol-related behaviors. *Neuroscience & Biobehavioral Reviews*.

3. Sparta, D. R., Ferraro III, F. M., Fee, J. R., Knapp, D. J., Breese, G. R., & Thiele, T. E. (in press). The alcohol deprivation effect (ADE) in C57BL/6J mice is observed using operant self-administration procedures and is modulated by CRF-1 receptor signaling. *Alcoholism: Clinical & Experimental Research*.

4. Lyons, A. M., Lowery, E. G., Sparta, D. R., & Thiele, T. E. (2008). Effects of food availability and administration of orexigenic and anorectic agents on elevated ethanol drinking associated with drinking in the dark procedures. *Alcoholism: Clinical & Experimental Research*, *32,* 1962-1968.

5. Navarro, M., Cubero, I., Knapp, D. J., Breese, G. R., & Thiele, T. E. (2008). Decreased immunoreactivity of the melanocortin neuropeptide α-melanocyte stimulating hormone (α-MSH) after chronic ethanol exposure in Sprague-Dawley rats. *Alcoholism: Clinical & Experimental Research, 32*, 266-276.

6. Sparta, D. R., Sparrow, A. M., Lowery, E. G., Fee, J. R., Knapp, D. J., & Thiele, T. E. (2008). Blockade of the corticotropin releasing factor (CRF) type 1 receptor attenuates elevated ethanol drinking associated with drinking in the dark procedures. *Alcoholism: Clinical & Experimental Research, 32,* 259-265.

7. Lowery, E. G., Sparrow, A. M., Breese, G. R., Knapp, D. J., & Thiele, T. E. (2008). The CRF-1 receptor antagonist, CP-154,526, attenuates stress-induced increases in ethanol consumption by BALB/cJ mice. *Alcoholism: Clinical & Experimental Research,32,* 240-248.

CONFERENCE PRESENTATIONS

1. Knapp, D. H., Navarro, M., Huang, M., Wills, T. A., Overstreet, D. H., Thiele, T. E. & Breese, G. R. (2008). α-MSH prevents sensitization of withdrawal-induced anxiety-like behavior induced by the lipopoly-saccharide (LPS)/ethanol withdrawal. *Alcoholism: Clinical & Experimental Research, 32,* 80A.

2. Thiele, T. E., Knapp, D. J., Navarro, M., Overstreet, D. H., Breese, & G. R., McCown, T. J. (2008). Amygdalar transduction by a rAAV vector causing constitutive secretion of NPY blocks the alcohol deprivation effect and anxiety-like behavior in Alcohol Preferring P rats. *Alcoholism: Clinical & Experimental Research, 32,* 81A.

3. Sparrow, A. M., Lowery, E. G., & Thiele, T. E. (2008). NPY Y1 receptor knockout mice show increased sensitivity to stress-induced increases of ethanol intake and withdrawal-induced anxiety-like behavior. *Alcoholism: Clinical & Experimental Research, 32,* 33A.

4. Navarro, M., Cubero, I., & Thiele, T. E. (2008). Mutant mice lacking normal production of agouti-related protein (AgRP) show reduced operant self-administration and consumption of ethanol. *Alcoholism: Clinical & Experimental Research, 32,* 147A.

5. Lowery, E. G., Sparrow, A. M. & Thiele, T. E. (2008). The effects of stress on ethanol consumption in Balb/cJ, DBA/2J, and C57BL/6J mice. *Alcoholism: Clinical & Experimental Research, 32,* 32A.

6. Hayes, D. M., McCown, T. J., Fee, J. R., Breese, G. R., Knapp, D. J., & Thiele, T. E. (2008). Elevated NPY in the nucleus accumbens core augments sensitivity to ethanol-induced locomotor activity in inbred DBA/2J mice. *Alcoholism: Clinical & Experimental Research, 32,* 93A.

7. Cubero, I., Navarro, M., & Thiele, T. E. (2008). Assessment of basal and ethanol-induced alterations of α-MSH and AgRP immunoreactivity in low and high ethanol consuming inbred strains of mice. *Alcoholism: Clinical & Experimental Research, 32,* 78A.

CONCLUSIONS: We have made significant progress towards the goals of this research proposal. We have shown that NPY signaling is protective against the effects of stress on excessive ethanol drinking, and we have demonstrated that overexpression a Y2 receptor agonist in the amygdala protects against excessive ethanol self-administration. Both of these

observations are consistent with our overall guiding hypothesis. We have also found that NPY signaling modulates sensitization to the locomotor stimulant effects of ethanol. So what does this mean? These results have important implications for possible pharmacological medical treatment of stress-related alcoholism and alcohol relapse. Pharmacological targets aimed at the NPY system may prove to be effective in treating alcoholism resulting from exposure to traumatic events and stemming from PTSD, and may prevent relapse behavior in abstinent individuals since stress is a primary cause of relapse. Thus, these findings may be considered of high relevance to the U.S. military.

APPENDICES:

- Figures 1-5. In figures, * indicates significant differences between groups at the $p < 0.05$ level.

- 5 paper (4 in print, 1 in press) that were supported by this grant.

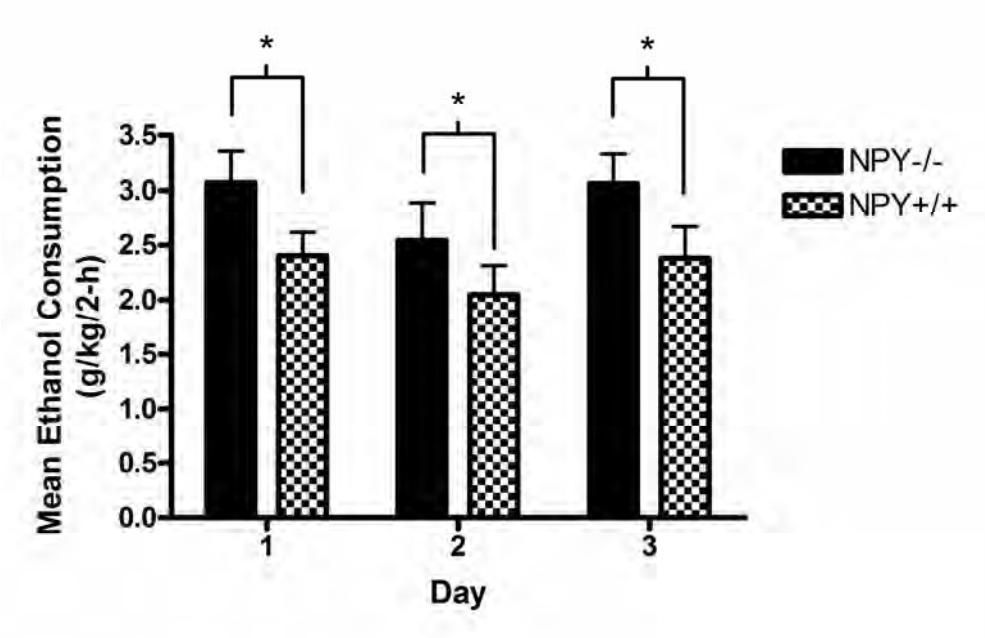

Figure 1

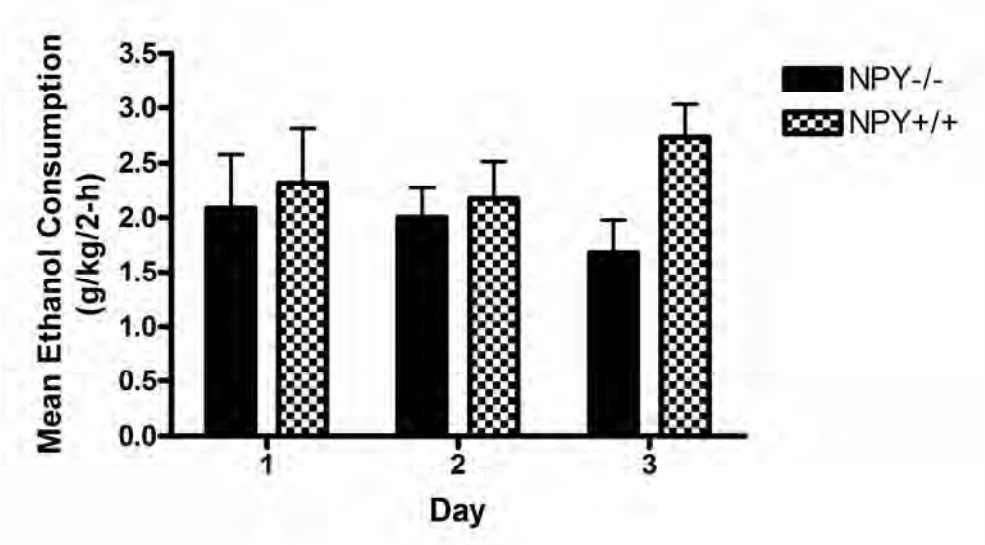

Figure 2

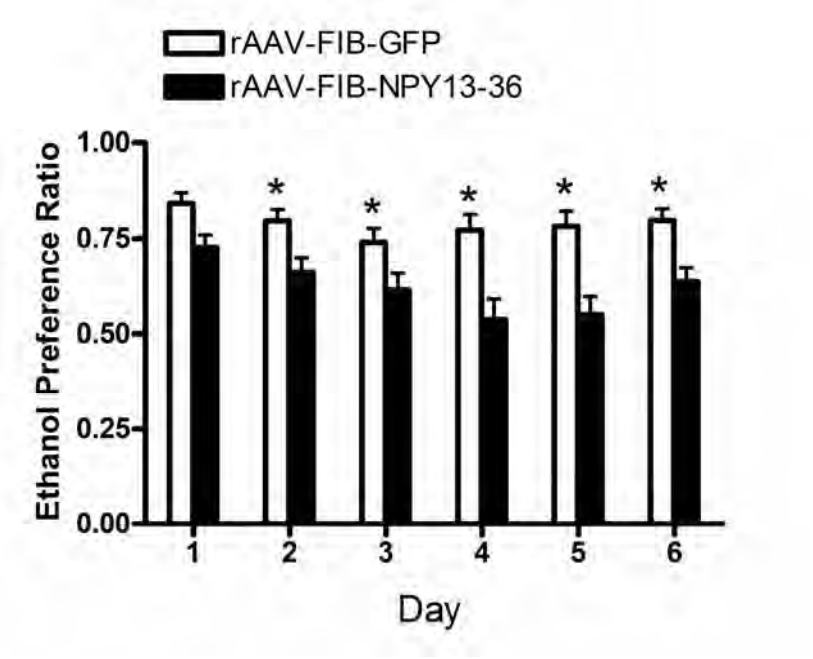
rAAV-FIB-GFP
rAAV-FIB-NPY13-36
Ethanol Preference Ratio
1.00
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Figure 3

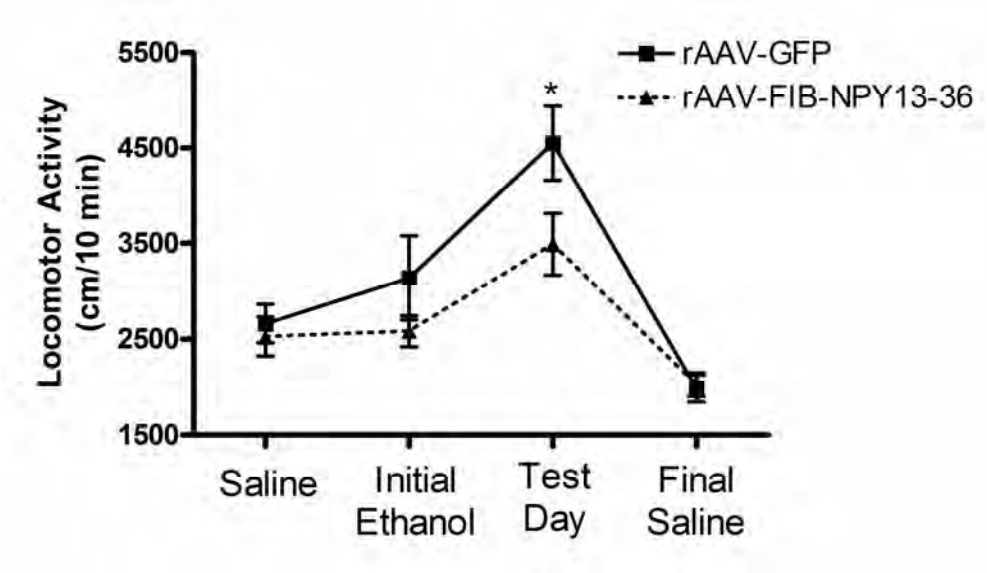

Figure 4

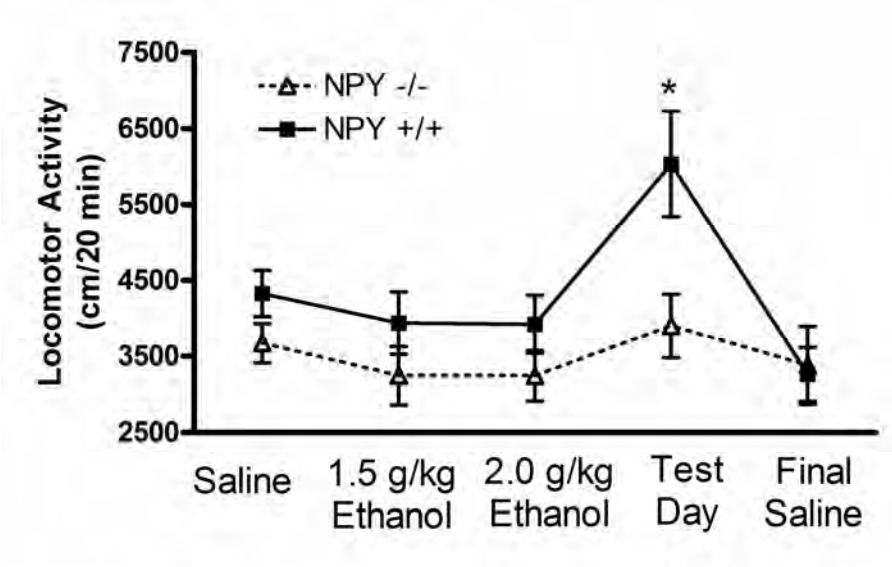

Figure 5

The CRF-1 Receptor Antagonist, CP-154,526, Attenuates Stress-Induced Increases in Ethanol Consumption by BALB/cJ Mice

Emily G. Lowery, Angela M. Sparrow, George R. Breese, Darin J. Knapp, and Todd E. Thiele

Background: Corticotropin-releasing factor (CRF) signaling modulates neurobiological responses to stress and ethanol, and may modulate observed increases in ethanol consumption following exposure to stressful events. The current experiment was conducted to further characterize the role of CRF_1 receptor (CRF_1R) signaling in stress-induced increases in ethanol consumption in BALB/cJ and C57BL/6N mice.

Methods: Male BALB/cJ and C57BL/6N mice were given continuous access to 8% (v/v) ethanol and water for the duration of the experiment. When a baseline of ethanol consumption was established, animals were exposed to 5 minutes of forced swim stress on each of 5 consecutive days. Thirty minutes before each forced swim session, animals were given an intraperitoneal injection of a 10 mg/kg dose of CP-154,526, a selective CRF_1R antagonist, or an equal volume of vehicle. The effect of forced swim stress exposure on consumption of a 1% (w/v) sucrose solution was also investigated in an ethanol-naïve group of BALB/cJ mice.

Results: Exposure to forced swim stress significantly increased ethanol consumption by the BALB/cJ, but not of the C57BL/6N, mice. Stress-induced increases in ethanol consumption were delayed and became evident approximately 3 weeks after the first stressor. Additionally, forced swim stress did not cause increases of food or water intake and did not promote delayed increases of sucrose consumption. Importantly, BALB/cJ mice pretreated with the CRF_1R antagonist showed blunted stress-induced increases in ethanol intake, and the CRF_1R antagonist did not influence the ethanol drinking of non-stressed mice.

Conclusions: The present results provide evidence that CRF_1R signaling modulates the delayed increase of ethanol consumption stemming from repeated exposure to a stressful event in BALB/cJ mice.

Key Words: Corticotropin-Releasing Factor, CRF_1 Receptor, Ethanol, Stress, Voluntary Consumption.

STRESS MAY BE a key contributor to the development of ethanol dependence and relapse (Breese et al., 2005; Koob, 2003). Stressful life events, such as those underlying post-traumatic stress disorder, are comorbid with ethanol abuse disorders and human laboratory studies show that stress increases the self-report of craving in abstinent alcoholics (Back et al., 2006; Breslau et al., 2003; Fox et al., 2007). Clinical research implicates stress in the relapse to pathological ethanol use in formerly abstinent alcoholics, perhaps as a means to self-medicate heightened anxiety and negative affect associated with withdrawal and abstinence from alcohol (Brady and Sonne, 1999; Breese et al., 2005; Kushner et al., 1994; Sinha, 2001).

Recent investigations show that stress can also impact ethanol consumption in animal models (Chester et al., 2004; Croft et al., 2005; Le et al., 2000; Little et al., 1999; Liu and Weiss, 2002; Sillaber et al., 2002). Various stress paradigms reliably elicit stress-induced increases in ethanol consumption, especially among low ethanol consuming animals (Chester et al., 2004; Croft et al., 2005; Little et al., 1999). For example, selectively bred ethanol non-preferring NP rats exposed to 10 days of restraint stress showed significant and enduring increases in ethanol consumption beginning approximately 2 weeks following the stress procedure, while ethanol preferring P rats showed only transient stress-induced increases in ethanol drinking immediately after the stress procedure (Chester et al., 2004). Additionally, 3 weeks of stress induced by daily saline injections (Little et al., 1999) or 5 consecutive days of social defeat stress (Croft et al., 2005), significantly increased ethanol consumption approximately 2 weeks after the stress procedure among C57BL/10 mice displaying initially low preference for ethanol. An interesting commonality among many animal studies that assess the effects of stress on ethanol intake is that the effects of stress on ethanol drinking are

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delayed, typically occurring weeks after stress exposure (Chester et al., 2004; Croft et al., 2005; Little et al., 1999).

Both ethanol and stress activate the hypothalamic-pituitary-adrenal (HPA) axis by inducing the release of corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), and glucocorticoids (Brady and Sonne, 1999). The relationship between ethanol and the HPA-axis appears to be bidirectional, as exogenous administration of CRF, ACTH, and glucocorticoids alter ethanol consumption (Bell et al., 1998; O'Callaghan et al., 2002; Thorsell et al., 2005). Given that neurobiological responses to both stress and ethanol exposure involve HPA-axis signaling, it is possible that the neurochemicals and hormones associated with the HPA-axis modulate stress-induced increases of ethanol consumption. One such candidate is CRF, a 41 amino acid polypeptide that integrates both neuroendocrine and behavioral responses to stress (Smith et al., 1998). CRF-containing neurons are expressed throughout the brain, including in regions implicated in neurobiological responses to ethanol such as the bed nucleus of the stria terminalis, the amygdala, and the lateral hypothalamus (Koob, 2003). Of the two G protein-coupled receptors, the CRF_1 receptor (CRF_1R) appears to be involved with the integrate emotional behavior while the CRF_2 receptor (CRF_2R) may modulate ingestive behaviors (Koob, 2003; Zorrilla and Koob, 2004; Zorrilla et al., 2004).

Corticotropin-releasing factor receptor signaling has been implicated in a variety of neurobiological responses to ethanol. For example, CRF receptor antagonists attenuate the anxiogenic effect of ethanol withdrawal (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2004; Rassnick et al., 1993), prevent excessive ethanol self-administration in dependent animals (Funk et al., 2007; Valdez et al., 2002), and block foot shock-induced reinstatement of ethanol-seeking behavior (Liu and Weiss, 2002). The CRF_1R also appears to be involved in stress-induced increases in ethanol consumption. Mutant mice lacking normal production of the CRF_1R displayed significantly greater ethanol consumption beginning approximately 2 weeks after a social defeat stress procedure, an effect that was not evident in normal wild-type mice. Subsequent exposure to forced swim stress further augmented ethanol consumption in CRF_1R knockout mice (Sillaber et al., 2002).

While the Sillaber et al. (2002) study provides genetic evidence suggesting a role for the CRF_1R in modulating stress-induced increases in ethanol consumption, the goal of the present experiment was to use a pharmacological approach to determine if pretreatment with the selective CRF_1R antagonist, CP-154,526, would buffer the effects of stress and thus attenuate the development of stress-induced increases in ethanol intake in BALB/cJ mice. Therefore, we predicted that (1) ethanol consumption would increase among animals with a history of stress exposure and (2) pretreatment with CP-154,526 would attenuate stress-induced increases in ethanol consumption among animals with a history of stress. BALB/cJ mice were chosen because this strain has been shown to have high sensitivity to the effects of stress on both behavioral and neurobiological measures (Crawley et al., 1997) and drinks low levels of ethanol (Belknap et al., 1993). We also assessed the effects of stress exposure on ethanol consumption by C57BL/6N mice, a strain that voluntarily consumes high amounts of ethanol (Belknap et al., 1993). Here we show that 5 consecutive days of exposure to a 5-minute forced swim stress procedure caused significant and delayed increases in voluntary ethanol consumption in BALB/cJ mice, an effect which was attenuated by pretreatments with the CRF_1R antagonist before each stress session. On the other hand, stress exposure did not alter ethanol intake by C57BL/6N mice.

MATERIALS AND METHODS

Animals

Forty-seven male BALB/cJ (Jackson Laboratories, Bar Harbor, ME) and 36 male C57BL/6N (Charles River Labs, Wilmington, MA) mice approximately 8-week old and weighing 19 to 26 g were housed individually in polypropylene cages with corncob bedding upon arrival. Animals had ad libitum access to tap water and standard rodent chow throughout the experiment. All fluid was presented in 2 bottles, inserted through holes at the top of the cage. Bottle weights were recorded every 2 days, and body weights and food measurements were taken every 4 days at approximately 10:00 AM. Food intake was measured by subtracting the weight of rodent chow (grams) still present in the cage on measurement day from the initial weight when food was placed in the cage. Great care was taken to collect the remaining food in the cage on measurement day to assure accurate readings. The colony room was maintained at approximately 21° C with a 12-h/12-h light/dark cycle with lights off at 10:30 AM. All procedures in the experiments below were approved by the University of North Carolina's Institutional Animal Care and Use Committee and follow the National Institute of Health's guidelines.

Drug Treatment

CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) donated by Pfizer (Groton, CT) was suspended in a vehicle of 0.5% carboxymethylcellulose (CMC). CP-154,526 displays high affinity for the CRF_1R (K_i < 10 nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al., 1996; Schulz et al., 1996). Peripheral administration of CP-154,526 crosses the blood–brain barrier and reaches peak brain concentrations 20 minutes after administration with significant levels of the drug observed in the cortex, striatum, cerebellum, and hippocampus (Keller et al., 2002). Importantly, previous research found that systemic administration of a 10 mg/kg dose of CP-154,526 effectively reduced anxiety-like behavior in mice (Griebel et al., 1998). Therefore, a 10 mg/kg dose of CP-154,526, or equal volume of CMC (5 ml/kg), was administered via intraperitoneal (i.p.) injection approximately 30 minutes prior to each stress or handling procedure (see below).

Forced Swim Stress

Forced swim procedures were used to induce stress in mice. Briefly, the mice were removed from their homecages and placed individually in buckets containing 4,000 ml of water maintained at approximately

room temperature (21°C) for 5 minutes on each of 5 consecutive days. Mice were carefully monitored and a criteria was established that any mouse that could not keep its head above the water was removed from the procedure (however, all animals were able to swim for the entire session in each experiment). After the 5-minute session, mice were removed from the buckets and dried with a cloth towel. This forced swim stress procedure has been shown to significantly increase ethanol drinking by mice (Sillaber et al., 2002). Mice in the non-stress conditions were briefly removed and then returned to their cages.

Habituation to Environment and Voluntary Ethanol Consumption

Upon arrival, animals were allowed to habituate to their surroundings for 8 days. On day 9, 1 water bottle on each cage was replaced with an identical bottle containing a 2% (v/v) ethanol solution diluted in tap water. Every 4 days, the concentration of ethanol was increased in the following increments: 4, 6, and 8%. From this point on, animals had continuous free access to 8% ethanol and water for the duration of the experiment. The position of bottles containing ethanol were changed every 2 days to prevent the development of side preferences. Fluid loss was controlled by using dummy bottles of water and ethanol placed on an animal-free cage which was located on the same rack as cages containing mice. Daily ethanol consumption was calculated in grams of ethanol consumed/kg of body weight (g/kg).

Consumption of the 8% ethanol solution stabilized by day 13, and animals were divided into 4 groups based on ethanol consumption during the final 3 days of baseline (days 16 to 18). Mice were either pretreated with CP-154,526 (CP) or vehicle (Veh) 30 minutes before being exposed to a 5-minute forced swim stress session (Stress) or handling (No Stress). The groups were as follows: BALB/cJ Stress-CP ($n = 8$), BALB/cJ Stress-Veh ($n = 8$), BALB/cJ No Stress-CP ($n = 9$), BALB/cJ No Stress-Veh ($n = 9$), C57BL/6N Stress-CP ($n = 10$), C57BL/6N Stress-Veh ($n = 7$), C57BL/6N No Stress-CP ($n = 9$), and C57BL/6N No Stress-Veh ($n = 10$). Following the 5-forced swim days, ethanol, water, and food intake as well as body weight measures were collected over a 4-week period. The BALB/cJ mice were exposed to an additional 5 days of forced swim stress on days 56 to 60, as described above, but did not receive drug treatment prior to stress exposure.

Voluntary Sucrose Consumption and Forced Swim Stress

As a consummatory control, 20 ethanol-naïve BALB/cJ mice were given continuous access to a 1% (w/v) sucrose solution and tap water and exposed to forced swim stress or handling, as described above. Sucrose was diluted in tap water. We chose 1% sucrose because we found that this concentration produced a similar volume of consumption by the BALB/cJ mice as the 8% ethanol solution. Additionally, 1% sucrose solution has been used previously as a control for stress-induced consumption of an 8% ethanol solution (Croft et al., 2005). The position of bottles containing sucrose was changed every 2 days to prevent the development of side preferences. Fluid loss was controlled by using dummy bottles of water and sucrose placed on an animal-free cage which was located on the same rack as cages containing mice. Daily sucrose consumption was calculated in milliliters of sucrose solution consumed/kg of body weight (ml/kg). Access to food, water, and sucrose was continuously available for the duration of the experiment.

Following 7 days of access to the 1% sucrose solution, animals were divided into Stress and No Stress groups based on their sucrose consumption during the final 3 days of baseline (days 5 to 7). On days 8 through 12, animals in the Stress group ($n = 10$) were exposed to daily 5-minute forced swim procedures over 5 days, while animals in the No Stress group ($n = 10$) were handled as described above. Sucrose and water consumption were monitored every 2 days throughout the stress period, and for an additional 4 weeks thereafter.

Data Analyses

All data shown are presented as means ± SEM and were analyzed using repeated measures analyses of variance (ANOVAs). Planned comparisons were analyzed using *t*-tests (Winer, 1991). In accordance with a priori hypotheses, the following tests were conducted: (1) comparisons were made of the Stress-Veh and No Stress-Veh groups to determine if stress exposure significantly increased ethanol consumption, (2) comparisons were made of the Stress-CP group with No Stress-CP and No Stress-Veh groups to determine if CP-154,526 pretreatment significantly attenuated stress-induced ethanol drinking to the level of non-stressed animals, and (3) comparisons were made of the Stress-Veh and Stress-CP groups to determine if CP-154,526 pretreatment significantly blocked stress-induced increases of ethanol drinking relative to stressed animals not pretreated with the CRF_1R antagonist. All reports of significance were accepted at the $p < 0.05$ level.

RESULTS

Figure 1 displays the effect of forced swim stress on the ethanol, water, and food consumption of BALB/cJ animals for the duration of the experiment. Because BALB/cJ mice were treated with the CRF_1R antagonist during the first, but not second, 5 day stress procedure, data were collapsed across the CRF_1R antagonist factor for the present analyses. As shown in Fig. 1*A*, forced swim stress significantly increased ethanol consumption among BALB/cJ animals in the Stress group, while handling did not alter ethanol consumption among BALB/cJ animals in the No Stress group. The results of a 2×11 repeated measures ANOVA revealed a significant main effect of week [$F(10,340) = 4.859$], a significant stress × week interaction [$F(10,340) = 2.634$], as well as a significant main effect of stress [$F(1,34) = 8.315$]. Planned comparisons revealed that stressed animals consumed significantly more ethanol than non-stressed animals at post-stress week 3 [$t(34) = 2.503$] and post-stress week 4 [$t(34) = 2.697$] following the first stressor. Additionally, stressed animals consumed significantly more ethanol during the second baseline period [$t(34) = 2.271$], during the second stress period [$t(34) = 1.971$], and at post-stress week 1 [$t(34) = 2.001$], post-stress week 2 [$t(34) = 2.378$], and post-stress week 3 [$t(34) = 2.845$] following the second stressor. Animals of the Stress group consumed significantly less water when compared with animals of the No Stress group for much of the experiment (see Fig. 1*B*). The results of a 2×11 repeated measures ANOVA revealed a significant main effect of week [$F(10,340) = 5.750$] and a significant stress × week interaction [$F(10,340) = 3.342$]. Planned comparisons revealed that animals of the Stress group consumed significantly less water than animals of the No Stress group at post-stress week 4 following the first stressor [$t(34) = 2.423$] and following the second stressor at post-stress week 1 [$t(34) = 1.733$], post-stress week 2 [$t(34) = 2.234$], and post-stress week 3 [$t(34) = 1.727$]. The decrease in water consumption among

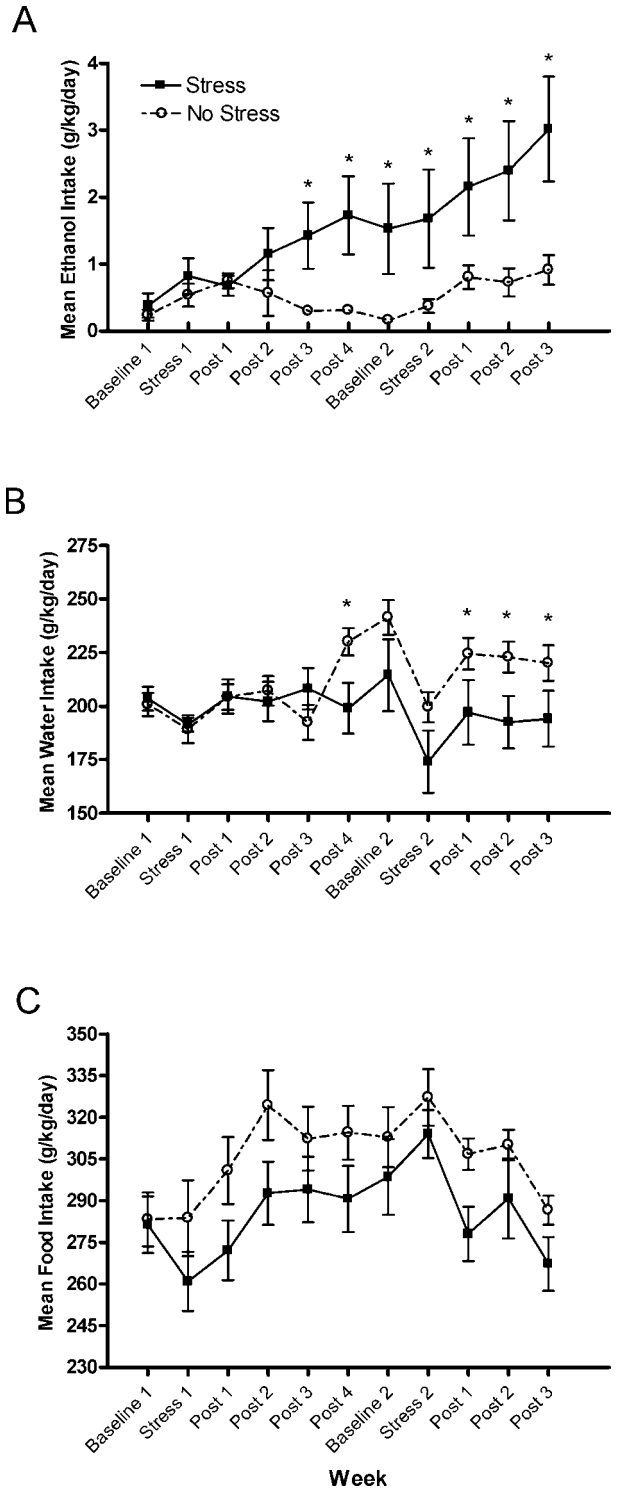

Fig. 1. Mean consumption (g/kg/d) of (**A**) ethanol, (**B**) water, and (**C**) food during baselines, the first and second stressors, and post-stress periods for BALB/cJ Stress and No Stress groups. All values are means ± SEM and *denotes significant between-group differences at the $p < 0.05$ level.

stressed animals is likely related to increased ethanol consumption following stress exposure. Finally, forced swim stress did not alter food consumption when compared with the handled group (see Fig. 1*C*), although a 2×11 repeated measures ANOVA revealed a significant main effect of week [$F(10,320) = 7.162$].

Figure 2 shows the effect of CRF_1R antagonism on ethanol, water, and food consumption of BALB/cJ animals during the first stress period. As shown in Fig. 2*A*, forced swim stress significantly increased ethanol consumption, an effect which was attenuated by administration of CP-154,526. The results of a $2 \times 2 \times 6$ repeated measures ANOVA indicated a significant stress × week interaction [$F(5,160) = 2.979$] as well as a significant main effect of stress [$F(1,32) = 17.986$]. Planned comparisons revealed that animals of the Stress-Veh group consumed significantly more ethanol than animals of the No Stress-Veh groups at post-stress week 3 [$t(16) = 2.046$] and post-stress week 4 [$t(16) = 1.963$], indicating stress-induced increases of ethanol consumption. Importantly, at no time point did group Stress-CP differ significantly from the non-stressed groups.

As stress-induced increases in ethanol consumption emerged several weeks following the stress procedure, the effects of CRF_1R antagonism on the development of stress-induced increases in ethanol consumption were analyzed by examining ethanol consumption at post-stress weeks 2 to 4 relative to the first week following the stress procedure (Δ post 1; see Fig. 2*B*). The results of a $2 \times 2 \times 3$ repeated measures ANOVA revealed a significant main effect of stress [$F(1,32) = 12.232$]. Planned comparisons revealed that animals of the Stress-Veh group showed significantly greater increases of ethanol consumption compared with the No Stress-Veh group at post-stress week 3 [$t(16) = 2.293$] and post-stress week 4 [$t(16) = 2.249$], again reflecting a delayed stress-induced increase in ethanol consumption. A planned comparison revealed significant differences between the Stress-Veh and Stress-CP groups at post-stress week 2 [$t(14) = 1.782$], suggesting that CP-154,526 blocked stress-induced increases in ethanol consumption during this week. As above, at no time point did the Stress-CP group differ significantly from the non-stressed groups.

Exposure to forced swim stress significantly altered water consumption, as displayed in Fig. 2*C*. The results of a $2 \times 2 \times 6$ repeated measures ANOVA revealed a significant main effect of week [$F(5,160) = 5.514$] as well as a significant stress × week interaction [$F(5,160) = 2.853$]. Planned comparisons revealed that the Stress-Veh group consumed significantly less water than the No Stress-Veh group at post-stress week 4 [$t(16) = 2.026$]. Finally, neither forced swim stress nor antagonism of the CRF_1R altered food consumption (see Fig. 2*D*). However, a significant main effect of week was observed [$F(5,160) = 7.486$].

Figure 3 shows the effects of forced swim stress on consumption of the 1% sucrose solution and water by ethanol-naive BALB/cJ mice. Repeated measures ANOVA did not reveal significant effects of stress on sucrose consumption when expressed as ml/kg/d or change in consumption relative to post-stress week 1. However, planned comparisons revealed significant differences in sucrose consumption

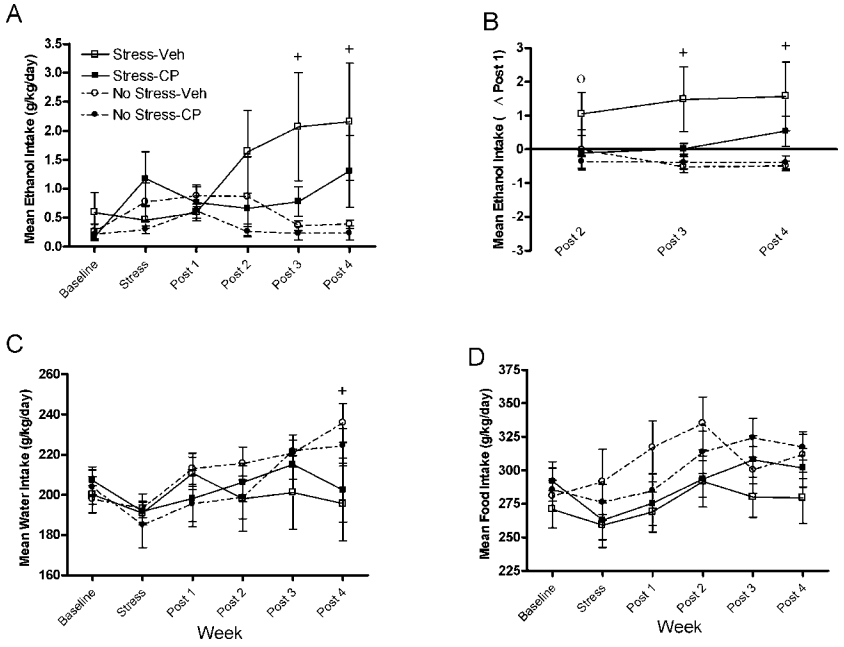

Fig. 2. (**A**) Mean ethanol consumption (g/kg/d) during the first baseline, stressor, and post-stress periods for BALB/cJ mice. (**B**) Mean changes in ethanol consumption (g/kg/d) during post-stress weeks 2 to 4 relative to post-stress week 1 during the first post-stress period for BALB/cJ mice. (**C**) Mean water consumption (g/kg/d) during the first baseline, stressor, and post-stress periods for BALB/cJ mice. (**D**) Mean food consumption (g/kg/d) during the first baseline, stressor, and post-stress periods for BALB/cJ mice. Groups are as follows: Stress-Veh = mice pretreated with vehicle prior to forced swim exposure; Stress-CP = mice were pretreated with CP-154,526 prior to forced swim exposure; No Stress-Veh = mice were treated with vehicle and handled; No Stress-CP = mice were treated with CP-154,526 and handled. All values are means ± SEM. The high degree of variance noted in group Stress-Veh reflects an increase of random variation. Significant between group differences are as follows: ○denotes significant differences between the Stress-Veh and Stress-CP groups and +denotes significant differences between the Stress-Veh and No Stress-Veh groups, at the $p < 0.05$ level.

between groups. Specifically, as shown in Fig. 3*A*, significant differences in sucrose consumption were observed in stressed animals when compared with non-stressed animals at post-stress week 3 [$t(17) = 1.884$], and at post-stress week 4 [$t(17) = 2.139$], which appears to reflect a reduction of sucrose consumption by non-stressed mice at post-stress weeks 3 and 4 relative to prior weeks. Importantly, forced swim stress did not cause a delayed increase in sucrose consumption at post-stress weeks 2 to 4 relative to post-stress week 1 (Δ post 1). The effects of forced swim stress exposure on water consumption are shown in Fig. 3*C*. A 2×6 repeated measures ANOVA revealed a significant main effect of week [$F(5,85) = 6.237$], and planned comparisons revealed that the stressed animals consumed significantly less water than non-stressed animals at post-stress week 3, [$t(17) = 1.829$].

Figure 4 displays the effects of forced swim stress and CRF_1R antagonism on the ethanol and water consumption of C57BL/6N animals. As shown in Fig. 4*A*, neither forced swim stress nor CRF_1R antagonism significantly altered ethanol consumption by C57BL/6N animals. A $2 \times 2 \times 6$ repeated measures ANOVA revealed a significant main effect of week [$F(5,160) = 20.425$]. Planned comparisons revealed no group differences. Figure 4*B* shows water consumption by C57BL/6N mice. The results of a $2 \times 2 \times 6$ repeated measures ANOVA revealed a significant main effect of week [$F(5,160) = 7.087$], as well as a significant week × stress × drug interaction [$F(5,160) = 2.561$]. Planned comparisons revealed that animals of the Stress-Veh group consumed significantly more water than animals of the No Stress-Veh group at post-stress week 1 [$t(17) = 1.789$].

DISCUSSION

The results of the current experiment show that forced swim stress induced a delayed increase in ethanol consumption by initially low ethanol consuming BALB/cJ mice, but did not affect ethanol consumption in the initially high ethanol consuming C57BL/6N mice. The lack of effect of stress exposure on ethanol consumption by the C57BL/6N mice is unlikely due to the high baseline ethanol consumption observed in these animals (e.g., a ceiling effect) as experimental manipulations, such as procedures that promote the alcohol deprivation effect, have been shown to reliably increase ethanol consumption significantly above baseline levels which are similar to consumption levels observed in the present experiment (Melendez et al., 2006). These results are consistent with the literature suggesting that a variety of stressors can have delayed effects on ethanol consumption in rodents (Chester et al., 2004; Croft et al., 2005; Little et al., 1999; Sillaber et al., 2002), and that the effects of stress on ethanol consumption may depend on initial preference for ethanol (Chester et al., 2004; Little et al., 1999; Rockman et al., 1987). The results of the current experiment also provide additional support for research suggesting that CRF_1R signaling is

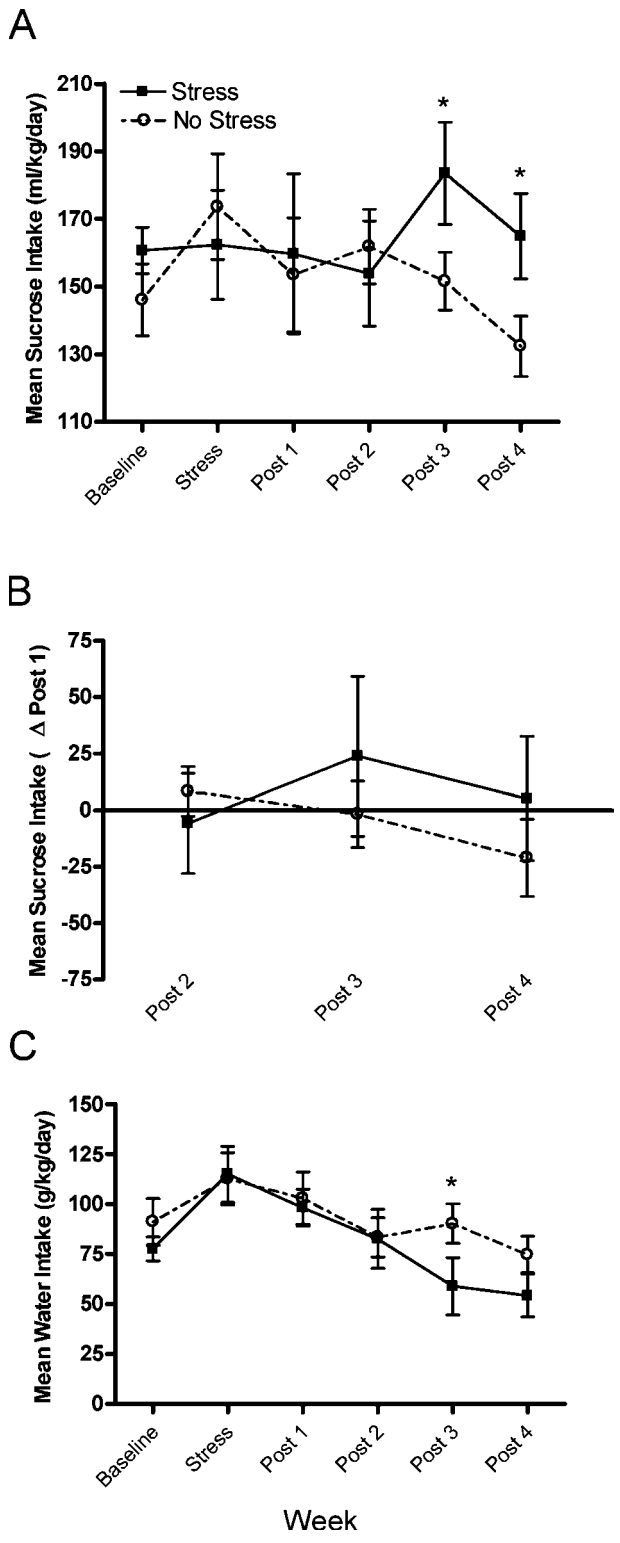

Fig. 3. (**A**) Mean consumption (ml/kg/d) of a 1% (w/v) sucrose solution during the baseline, stress, and post-stress periods for BALB/cJ Stress and No Stress groups. (**B**) Mean change in sucrose consumption (ml/kg/d) during post-stress weeks 2 to 4 relative to post-stress week 1 for BALB/cJ Stress and No Stress groups. (**C**) Mean water consumption (g/kg/d) during the baseline, stress, and post-stress period for BALB/cJ Stress and No Stress groups. All values are means ± SEM, and *denotes significant differences between the Stress and No Stress groups, at the $p < 0.05$ level.

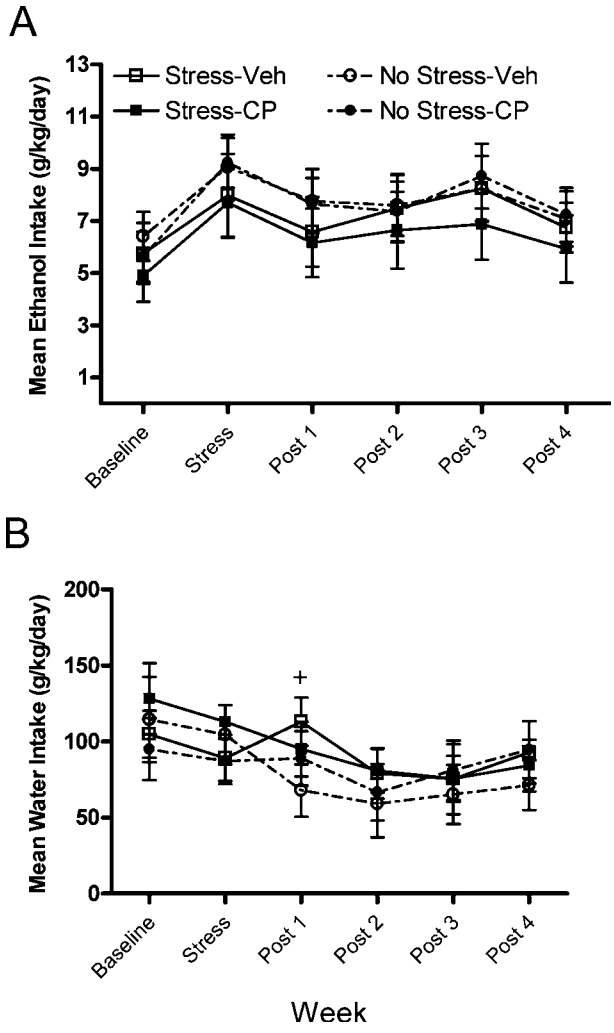

Fig. 4. (**A**) Mean consumption (g/kg/d) of ethanol during the baseline, stress, and post-stress periods for C57BL/6N mice. (**B**) Mean water consumption (g/kg/d) during the baseline, stress, and post-stress periods for C57BL/6N mice. Groups are as follows: Stress-Veh = mice pretreated with vehicle prior to forced swim exposure; Stress-CP = mice were pretreated with CP-154,526 prior to forced swim exposure; No Stress-Veh = mice were treated with vehicle and handled; No Stress-CP = mice were treated with CP-154,526 and handled. All values are means ± SEM, and $^{+}$denotes significant differences between the Stress-Veh and No Stress-Veh groups at the $p < 0.05$ level.

involved in stress-related ethanol consumption as pretreatment before each stress episode with CP-154,526, a CRF_1R antagonist, attenuated the observed stress-induced increases in ethanol consumption among BALB/cJ mice. This conclusion is supported by the observation that stress-treated BALB/cJ mice that were pretreated with CP-154,526 never differed significantly in ethanol consumption from non-stressed groups, while stress-treated mice pretreated with the vehicle showed significantly higher levels of ethanol consumption than the non-stressed groups at multiple time points.

Although there were group differences in sucrose consumption, such differences appear to be related, in part, to a reduction of sucrose intake by non-stressed mice at post-stress weeks 3 and 4 relative to prior weeks. Furthermore, there

were no group differences in sucrose consumption at post-stress weeks 2 through 4 relative to post-stress week 1, indicating that stress did not promote a delayed increase of sucrose consumption, a delayed effect of stress that was noted when mice drank ethanol. This observation, and the fact that stress did not significantly alter food intake, suggests that the delayed effect of stress to increase consumption over weeks is specific to ethanol. The observed decrease in water consumption among animals exposed to stress is likely related to the observed increase in ethanol solution intake among these animals, as a portion of the animal's water intake was obtained from the ethanol solution.

Although the literature on stress and ethanol consumption has been mixed, recent reports indicate that the effects of stress on ethanol consumption may differ depending on the length of time that has elapsed since termination of the stressor. For example, some studies investigating the immediate effects of stress on ethanol consumption suggest that ethanol consumption is transiently reduced (van Erp and Miczek, 2001), and some studies investigating the long-term effects of stress on ethanol consumption reveal delayed increases in ethanol consumption (Chester et al., 2004; Croft et al., 2005; Sillaber et al., 2002), though other studies have failed to find a stress effect on ethanol consumption at any experimental time point (Bowers et al., 1997; Boyce-Rustay et al., 2007). Indeed, direct comparison of the results of these studies is difficult due to use of a wide variety of stressors and rodent strains, as well as varying experimental time points and ethanol access periods. Nonetheless, our work and the work of others indicate that stress can increase ethanol consumption by rodents under certain conditions.

The results of the current experiment coincide with an increasing number of reports suggesting that the pattern of ethanol consumption following stress may be dependent on predisposed ethanol preference (Chester et al., 2004; Little et al., 1999; Rockman et al., 1987), as increases in ethanol consumption were observed in initially low ethanol consuming BALB/cJ mice approximately 3 weeks after exposure to forced swim stress, but not in initially high ethanol consuming C57BL/6N mice. Prior research suggests that animals genetically predisposed, or phenotypically selected, for high ethanol consumption, such as the C57BL/6 strain of mice, reduce ethanol consumption during stress exposure and gradually return to baseline levels of consumption after termination of the stressor (Chester et al., 2004; Rockman et al., 1987). For example, ethanol preferring P rats displayed significantly reduced ethanol consumption during the first 5 days of exposure to 10 days of unpredictable restraint stress, an increase in ethanol consumption during the 5 days immediately following the termination of the restraint stress, and a subsequent return to baseline levels of ethanol consumption (Chester et al., 2004). Similarly, Wistar rats screened for high ethanol preference and exposed to unpredictable restraint stress at cold temperatures significantly reduced their ethanol consumption during the first 12 days of an 18-day stress period, after which consumption returned to baseline levels (Rockman et al., 1987).

Conversely, a variety of observations reveal that animals showing initial low ethanol preference, such as the BALB/c strain of mice, continue consuming baseline levels of ethanol during, and immediately following stress exposure, but increase levels of ethanol consumption approximately 2 to 3 weeks following termination of the stressor (Chester et al., 2004; Croft et al., 2005; Rockman et al., 1987). Consistently, ethanol non-preferring NP rats exposed to 10 days of unpredictable restraint stress maintained baseline levels of ethanol consumption throughout the stress period and immediately thereafter, and significantly increased ethanol consumption approximately 2 weeks following stress exposure (Chester et al., 2004). Wistar rats screened for low ethanol preference and exposed to 18 days of unpredictable restraint stress at cold temperatures displayed gradual increases in ethanol consumption beginning in the final 12 days of the stress period and continuing several weeks after the stress exposure (Rockman et al., 1987). Similar delayed increases in ethanol consumption have been observed in C57BL/10 mice screened for low ethanol preference and exposed to social defeat stress (Croft et al., 2005), and stress caused by repeated saline injections (Little et al., 1999; O'Callaghan et al., 2002). Thus, an emerging literature provides converging evidence that a variety of stressors induce delayed increases in ethanol consumption in initially low ethanol consuming animals. While the present observations provide additional evidence that stress-induced increases in ethanol drinking are evident in low (BALB/cJ), but not high (C57BL/6N), ethanol preferring strains, an alternative explanation for the present data is that the BALB/cJ mice were more stress-responsive than the C57BL/6N mice. Indeed, a well-established literature suggests that the BALB/c strain of mice display higher levels of anxiety and are more stress-responsive on certain behavioral measures than the C57BL/6 strain of mice (Anisman et al., 2007; Carola et al., 2002; Crawley et al., 1997; Depino and Gross, 2007; Ducottet and Belzung, 2004; Griebel et al., 2000). As such, it may be stress sensitivity, rather than initial ethanol preference, that predicts the effects of stress on subsequent ethanol intake.

The HPA-axis has been implicated in neurobiological responses to stress and ethanol consumption, and the involvement of neurochemicals and hormones associated with the HPA-axis in stress-induced ethanol consumption has been demonstrated. For example, Sprague–Dawley rats with intact HPA-axis function displayed increases in ethanol consumption following 11 days of unpredictable exposure to either isolation or immobilization stress, while the post-stress ethanol consumption of hypophysectomized rats did not change (Nash and Maickel, 1988). Pharmacological manipulations also provide evidence for a role of HPA-axis signaling. ACTH administered via unpredictable, i.v. injections for 11 days in intact rats produced increases in ethanol consumption similar to those observed following stress exposure (Nash and Maickel, 1988). Mice screened for low ethanol preference and given

3 weeks of daily i.p. injections of the corticosterone synthesis inhibitor metyrapone did not display stress-induced increases in ethanol preference caused by repeated i.p. injection, while mice injected with vehicle over 3 weeks did display increases in ethanol preference (O'Callaghan et al., 2002). The Type II glucocorticoid receptor appears to modulate the effects of corticosterone on stress-induced increases in ethanol consumption as mice screened for low ethanol preference and given daily i.p. injections of the glucocorticoid Type II receptor antagonist RU38486 did not display stress-induced increases in ethanol preference, an effect observed in mice with low ethanol preference and given daily i.p. injections of vehicle (O'Callaghan et al., 2002).

The results of the current experiment, as well as those of Sillaber et al. (2002), indicate that CRF signaling, via the CRF_1R, is another HPA-axis-associated neurochemical that modulates stress-induced ethanol consumption. In the current experiment, the role of the CRF_1R was investigated pharmacologically through the administration of the CRF_1R antagonist CP-154,526 prior to each exposure to forced swim stress. While only 1 dose of the CRF_1R antagonist was used in the present study, this 10 mg/kg dose of CP-154,526 has been previously shown to reduce anxiety-like behavior in BALB/cJ mice (Griebel et al., 1998). Importantly our results indicate that pharmacological antagonism of the CRF_1R with a 10 mg/kg dose of CP-154,526 attenuates the delayed stress-induced increases in ethanol consumption observed in vehicle and stress treated animals. On the other hand, Sillaber et al. (2002) found that disruption of CRF_1R signaling by genetic mutation augmented the delayed stress-induced increases of ethanol consumption relative to wild-type mice. While the factors that contribute to the inconsistencies between pharmacological and genetic manipulation of CRF_1R signaling are not completely clear, Sillaber et al. (2002) suggest that the observed increases in ethanol consumption among CRF_1R knockout mice following stress exposure may result from developmental compensation associated with mutation of the CRF_1R gene. It should be noted that although the results of the current experiment suggest that the CRF_1R modulates stress-related ethanol consumption, it remains unclear if CRF_1R signaling within the HPA-axis and/or within extrahypothalamic brain regions are involved. In fact, a recent report found that pretreatment with the CRF_1R antagonist antalarmin attenuated yohimbine-induced increases in ethanol self-administration in rats without altering yohimbine-induced increases of corticosterone levels, suggesting that extrahypothalamic CRF_1R signaling was involved (Marinelli et al., 2007).

In summary, the current experiment indicates that exposure to stress is associated with delayed increases in ethanol consumption among initially low consuming BALB/cJ mice, but not initially high consuming C57BL/6N mice. Importantly, stress did not alter the consumption of food or cause delayed increases of sucrose intake in BALB/cJ mice. Pretreatment before each stress episode with the CRF_1R antagonist CP-154,526 attenuated the delayed increases in ethanol consumption observed in stressed BALB/cJ mice, but did not alter the consumption of ethanol by non-stressed mice. Current research indicates that CRF signaling, via the CRF_1R, is intricately involved in the development of ethanol dependence and relapse to ethanol seeking during abstinence (Heilig and Koob, 2007), perhaps due to the role CRF plays in mediating increased anxiety during withdrawal from ethanol (Breese et al., 2004). The current experiment supports the hypothesis that CRF, and more specifically the CRF_1R, is also involved in delayed and long-lasting stress-induced increases in ethanol drinking. Thus targets aimed at the CRF_1R may be useful compounds for treating and/or preventing the lasting effects of stress exposure to induce excessive and uncontrolled ethanol consumption in the human population. Finally, future research will extend the current findings by investigating the role of CRF_1R signaling in targeted brain areas, as well as the role of CRF in stress-induced ethanol drinking by ethanol dependent animals.

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Effects of Food Availability and Administration of Orexigenic and Anorectic Agents on Elevated Ethanol Drinking Associated With Drinking in the Dark Procedures

Angela M. Lyons, Emily G. Lowery, Dennis R. Sparta, and Todd E. Thiele

Background: Drinking in the dark (DID) procedures have recently been developed to induce high levels of ethanol drinking in C57BL/6J mice, which result in blood ethanol concentrations reaching levels that have measurable affects on physiology and/or behavior. The present study determined if increased ethanol drinking associated with DID procedures may be motivated by caloric need rather than by the postingestive pharmacological effects of ethanol. To this end, food availability was manipulated or mice were given peripheral administration of orexigenic or anorectic agents during DID procedures.

Methods: C57BL/6J had 2-hours of access to the 20% (v/v) ethanol solution beginning 3-hours into the dark cycle on days 1 to 3, and 4-hours of access to the ethanol bottle on day 4 of DID procedures. In Experiment 1, the effects of food deprivation on ethanol consumption during DID procedures was assessed. In Experiments 2 and 3, mice were given intraperitoneal (i.p.) injection of the orexigenic peptide ghrelin (0, 10 or 30 mg/kg) or the anorectic protein leptin (0 or 20 μg/g), respectively, before access to ethanol on day 4 of DID procedures. In Experiment 4, hourly consumption of food and a 0.05% saccharin solution were assessed over a period of hours that included those used with DID procedures.

Results: Consistent with previous research, mice achieved blood ethanol concentrations (BECs) that ranged between 100 and 150 mg% on day 4 of DID experiments. Neither food deprivation nor administration of orexigenic or anorectic compounds significantly altered ethanol drinking with DID procedures. Interestingly, mice exhibited their highest level of food and saccharin solution consumption during hours that overlapped with DID procedures.

Conclusions: The present observations are inconsistent with the hypothesis that C57BL/6J mice consume large amounts of ethanol during DID procedures in order to satisfy a caloric need.

Key Words: C57BL/6J Mice, Drinking in the Dark, Food, Alcohol, Ghrelin, Leptin, Calories.

TO ASSIST IN identifying the genetic and neurobiological factors that underlie alcoholism, scientists often turn to animal models to address questions that cannot be ethically studied in human subjects. However, in many cases rodents do not consume enough alcohol to reach the point of behavioral and/or pharmacological intoxication (Spanagel, 2000). Recently, "drinking in the dark" (DID) procedures have been developed to induce excessive binge-like ethanol drinking in C57BL/6J mice, which result in blood ethanol concentrations (BECs) reaching levels that have measurable effects on physiology and/or behavior (Rhodes et al., 2005, 2007). DID procedures involve giving C57BL/6J mice access to a 20% ethanol solution for 2 to 4-hours starting 3-hours into their dark cycle. C57BL/6J can achieve BECs of >100 mg% and exhibit signs of behavioral intoxication as measured by motor deficits on the rotarod and balance beam (Rhodes et al., 2005, 2007). Pretreatment with naltrexone or the dopamine re-uptake inhibitor GBR 12909 attenuate increased ethanol consumption associated with DID procedures, suggesting a role for opioid and dopamine receptor signaling (Kamdar et al., 2007). More recently, we have found that pretreatment with the CRF_1 receptor antagonist CP-154,526 protected against high levels of ethanol drinking associated with specific DID procedures. Importantly, CRF_1 receptor blockade did not alter ethanol drinking in C57BL/6J mice consuming moderate amounts of ethanol, suggesting that CRF_1 receptor signaling specifically modulates high ethanol intake (Sparta et al., 2008).

As DID procedures involve providing C57BL/6J mice with access to ethanol towards the beginning of the dark

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cycle, increased ethanol drinking may be related to the high level of nocturnal ingestive behavior that is characteristic of mice (Ho and Chin, 1988; Tabarin et al., 2007). In fact, the highest levels of food intake in mice were found to occur during the first 4 hours of their 12 hour dark cycle (Tabarin et al., 2007), a window of time in which mice are given access to ethanol using DID procedures (Kamdar et al., 2007; Rhodes et al., 2005, 2007). Since ethanol contains calories, this raises the possibility that increased ethanol drinking associated with DID procedures results from presenting ethanol during a time of high caloric need, rather than an increased motivation to drink ethanol for its postingestive pharmacological effects.

The experiments described below assessed the possibility that increased ethanol drinking with DID procedures stems from caloric need. In Experiment 1, we reasoned that if ethanol were the only source of calories during DID procedures, ethanol intake would be increased relative to mice with free access to food if the caloric need hypothesis is correct. To further test this caloric need hypothesis, in Experiment 2 mice were given intraperitoneal (i.p.) injection of ghrelin immediately before DID procedures. Ghrelin is an orexigenic gut peptide shown to increase food consumption when given peripherally to C57BL/6J mice (Wang et al., 2002). We predicted that if increased ethanol drinking resulting from DID procedures was related to increased caloric need, an orexigenic peptide such as ghrelin should further augment ethanol intake. In Experiment 3, we determined if the protein leptin would influence ethanol intake when given before DID procedures. Leptin is synthesized in adipose tissue and reduces food intake when administered peripherally (Halaas et al., 1995; Prpic et al., 2003). If increased ethanol intake with DID procedures stems from caloric need, we predicted that the anorectic protein leptin should decrease ethanol intake. In Experiment 4, we examined the normal consummatory behaviors of C57BL/6J mice with a caloric substance (standard rodent chow) and a noncaloric substance (0.05% saccharin solution) over a twelve-hour period, which included the timeframe that mice were given ethanol during DID procedures. Data from the experiments described below are inconsistent with the hypothesis that increased ethanol drinking stemming from DID procedures results from increased caloric need.

METHODS

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor ME) were used in all experiments. Mice were 6 to 8 weeks old when they arrived from the vendor and weighed between 25 and 30 g at the onset of the experiments. Mice were single housed in polypropylene cages with corncob bedding. Standard rodent chow (Teklad, Madison, WI) and water were available at all times except where noted. The vivarium rooms were maintained at an ambient temperature of 22°C with a reverse 12-h/12-h light-dark cycle. Lights came on at 9:00 PM and went off at 9:00 AM. Mice were acclimated to the environment for at least 10 days before the start of experiments. All experimental procedures were approved by the University of North Carolina Animal Care and Use Committee (IACUC) and were in compliance with the NIH Guide for Care and Use of Laboratory Animals.

Experiment 1: Effects of Food Availability on Ethanol Intake Associated With DID Procedures

Before the start of the experiment, mice were divided into groups (n = 10/group) based on bodyweight so that the average weight between groups was similar. All mice underwent a modified DID protocol (Rhodes et al., 2005; Sparta et al., 2008). Briefly, all homecage water bottles were replaced with a single bottle of 20% (v/v) ethanol, 3-hours into the start of the dark phase. The 20% ethanol solution remained on the homecage for 2-hours during the training sessions (days 1 to 3) and for 4-hours on the test day (day 4). For each of the 4-days during DID procedures, 1 group of mice had ad libitum access to food (no food deprivation), the second group of mice had food removed from their cages 3-hours before the beginning of the dark cycle, the third group of mice had food removed from their cages at the beginning of the dark cycle, and the fourth group of mice had food removed from their cages beginning 3-hours into the dark cycle. For all food deprived groups, food was returned 7-hours into the dark cycle. With this arrangement, mice were food deprived for 0, 6 to 8, 3 to 5, or 0 to 2 hours during DID procedures on days 1 to 3 and 0, 6 to 10, 3 to 7, or 0 to 4 hours during DID procedures on day 4. Immediately following the 4-hours of ethanol access on day 4, tail blood samples (6 μl) were collected from all mice to determine BECs with an alcohol analyzer (Analox Instruments, Lunenburg, MA). On each day of study, ethanol consumption and body weight measures were recorded and the amount of ethanol consumed was calculated as g of ethanol consumed per kg of body weight (g/kg).

Experiment 2: Effects of Ghrelin Injection on Ethanol Intake Associated With DID Procedures and Basal Food Consumption

The DID procedures were similar to those described above. Because ghrelin is a potent orexigenic agent that stimulates food intake when given peripherally (Chen et al., 2004; Wang et al., 2002), food was removed from all mice cages immediately before ethanol access on each of the 4-days of DID procedures. This was done to avoid the potential confound of altered ethanol drinking secondary to increased food intake. To habituate mice to i.p. injections, all mice received an i.p. injection of 0.9% saline (5 ml/kg) for 2 days prior to the start of the experiment and immediately before ethanol access on days 1 to 3. Mice were then distributed into 3-groups (n = 10/group) matched for average ethanol consumption that occurred over the first 3-days of the experiment (that is, the mice were distributed so that the baseline level of ethanol consumption was approximately equal between the groups). On the fourth day, mice were given an i.p. injection of 1 of 3 doses (0, 10, or 30 g/kg) of mouse ghrelin (Phoenix Pharmaceuticals, Inc., Burlingame, CA) mixed in 0.9% saline immediately before application of the ethanol bottle. The 30 g/kg dose of ghrelin has been shown to significantly increase 4-hour food intake by mice (Chen et al., 2004). Tail blood (6 μl) was collected from all mice immediately following the 4-hour test session to determine BECs. Ethanol consumption and body weight measures were recorded and the amount of ethanol consumed was calculated as g of ethanol consumed per kg of body weight (g/kg).

To confirm that ghrelin was physiologically active, a naïve group of C57BL/6J mice were used to assess the effects of ghrelin on food intake. Mice were distributed into 2-groups (n = 10/group) based on body weights. The mice received 2-days of habituation injections of 0.9% saline. On the test day, the food in each mouse cage was weighed at 3-hours into the dark cycle. At that time, mice were given

an i.p. injection of saline or ghrelin (30 mg/kg). Food was weighed 4-hour later and food consumption was calculated as g consumed over the 4-hour test.

Experiment 3: Effects of Leptin Injection on Ethanol Intake Associated With DID Procedures and Basal Food Consumption

The procedures were similar to those in Experiment 2. Because leptin reduces food intake, food was removed from mice cages just before i.p. injections to avoid any confounds associated with altered ethanol drinking secondary to changes in food intake. Just before the beginning of the dark cycle, mice received an i.p. injection of 15 mM HCl mixed with 7.5 mM NaOH in saline (16 ml/kg, the vehicle used for leptin) for 2-days prior to the start of the experiment and on days 1 to 3 of the experiment to habituate them to the injection procedures. Mice were then distributed into 2-groups ($n = 10$/group) matched for average ethanol consumption that occurred over the first 3-days of the experiment. On the fourth day, mice were given an i.p. injection of vehicle or mouse leptin (20 μg/g mixed in 0.9% saline containing 15 mM HCl and 7.5 mM NaOH; Calbiochem, San Diego, CA) immediately before lights out. We chose to give leptin at the beginning of the dark phase rather than just before ethanol access because the actions of this dose of leptin on food last for up to 24-hours (Prpic et al., 2003). Ethanol bottles were placed on the cages 3-hours into the dark cycle as per the DID schedule. Tail blood sample (6 μl) was collected from all mice immediately following the 4-hour test session to determine BECs. Ethanol consumption and body weight measures were recorded and the amount of ethanol consumed was calculated as g of ethanol consumed per kg of body weight (g/kg).

To confirm that leptin was physiologically active, a naïve group of C57BL/6J mice were used to assess the effects of leptin on food intake. Mice were distributed into 2-groups ($n = 10$/group) based on body weights. The mice received 2-days of habituation injections (i.p.) of the leptin vehicle at the beginning of the dark cycle. Mice were then given an i.p. injection of vehicle or mouse leptin (20 μg/g) immediately before lights out and food consumption was measured starting 3-hours into the dark cycle and continued for 4-hours. Food consumption was calculated as g/4-h.

Experiment 4: Hourly Measures of Food Intake and Saccharin Consumption

Naïve C57BL/6J mice ($n = 7$) were used to assess the consummatory patterns of food (caloric substance) or a 0.05% (w/v) saccharin solution (noncaloric substance). After habituation to the environment (2-weeks), food consumption was measured hourly starting 1-hour before the beginning of the dark cycle and continuing until 11-hours into the dark cycle (thus, 4-hours before and after the normal DID testing period). Food consumption was calculated as g consumed for each hourly measure. After food measures were complete, the same mice were given 4-days of 2 bottle choice access to water and a 0.05% saccharin solution to habituate them to the novel tastant. Following the habituation period, hourly measures of saccharin solution were assessed beginning 1-hour before the beginning of the dark cycle until 11 hours into the dark cycle. Saccharin consumption was calculated as g consumed per kg of body weight (g/kg) for each hourly measure.

Data Analysis

All data in this report are presented as means ± SEM. One-way analyses of variance (ANOVAs) were used to analyze ethanol consumption and BEC data from Experiments 1 to 3. Repeated measures ANOVAs with post hoc t-tests were used to analyze food and saccharin consumption in Experiment 4. Significance was accepted at $p < 0.05$ (2-tailed).

RESULTS

Experiment 1: Effects of Food Deprivation on Ethanol Intake Associated With DID Procedures

Ethanol consumption during the 2-hour access on days 1 to 3 of Experiment 1 are presented in top portion of Table 1. ANOVAs revealed no significant group differences in ethanol consumption during days 1 to 3. Ethanol consumption and blood ethanol concentration data from the 4-hour test day of Experiment 1 are presented in Fig. 1*A* and *B*, respectively. One-way ANOVAs performed on these data revealed no significant effect of food deprivation on ethanol consumption [$F(3, 36) = 1.77$, $p = 0.17$] or blood ethanol concentrations [$F(3, 36) = 1.44$, $p = 0.25$]. There was a significant correlation between the amount of ethanol consumed and blood ethanol levels [$R = 0.69$, $p < 0.001$].

Experiment 2: Effects of Ghrelin Injection on Ethanol Intake Associated With DID Procedures and Basal Food Consumption

Ethanol consumption during the 2-hour access on days 1 to 3 of Experiment 2 are presented in middle portion of Table 1. ANOVAs revealed no significant group differences in ethanol consumption during days 1 to 3. Ethanol consumption and blood ethanol concentration data from the 4-hour test day of Experiment 2 are presented in Fig. 2*A* and *B*, respectively. One-way ANOVAs performed on these data revealed that neither of the doses of ghrelin examined significantly altered ethanol consumption [$F(2, 22) = 1.06$, $p = 0.36$] or blood ethanol levels [$F(2, 22) = 1.10$, $p = 0.35$]. There was a significant correlation between the amount of ethanol consumed and blood ethanol concentrations [$R = 0.604$, $p = 0.001$]. The effects of ghrelin on food consumption are presented in Fig. 3*C*. Administration of the 30 mg/kg dose of ghrelin significantly increased 4-hour food consumption [$F(1, 16) = 21.30$, $p < 0.001$], verifying that this dose of ghrelin was physiologically active in C57BL/6J mice.

Table 1. Ethanol Consumption (g/kg/2-h) on days 1 to 3 (mean ± SEM) of Experiments 1 to 3

	Day 1	Day 2	Day 3
Experiment 1 (hour food deprivation)			
0	4.13 ± 0.33	3.07 ± 0.26	2.52 ± 0.30
4	4.02 ± 0.62	2.58 ± 0.27	1.68 ± 0.33
7	3.53 ± 0.27	2.45 ± 0.32	1.92 ± 0.26
10	3.36 ± 0.18	2.60 ± 0.27	2.31 ± 0.45
Experiment 2 (mg/kg ghrelin)			
0	2.25 ± 0.40	1.97 ± 0.27	2.36 ± 0.69
10	1.68 ± 0.45	2.13 ± 0.28	2.12 ± 0.28
30	2.35 ± 0.36	1.82 ± 0.27	1.62 ± 0.27
Experiment 3 (μg/g leptin)			
0	1.83 ± 0.37	2.94 ± 0.45	1.94 ± 0.41
20	1.94 ± 0.28	2.16 ± 0.26	2.57 ± 0.31

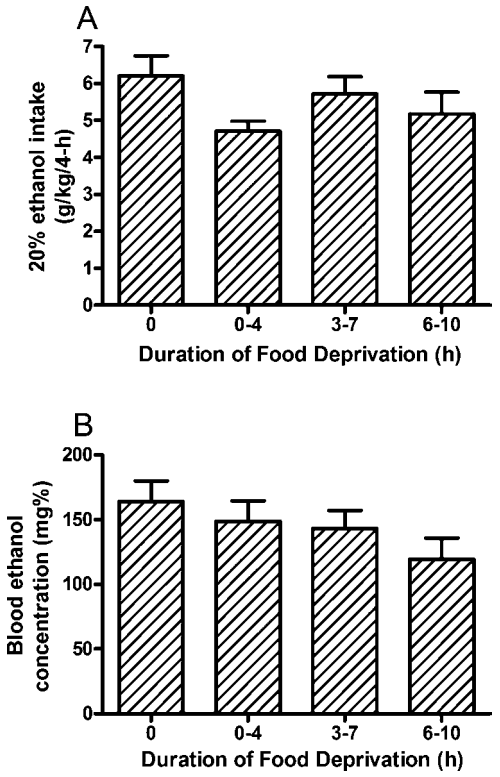

Fig. 1. Consumption of 20% (v/v) ethanol by C57BL/6J mice (**A**) and associated blood ethanol concentrations (**B**) during the 4-hour test day of Experiment 1. Mice were deprived of food for 0, 0 to 4, 3 to 7, or 6 to 10 hours during DID procedures on day 4, which corresponded to ad libitum access to food, or removing food at the initiation of DID procedures, 3-hours before DID procedures (at the beginning of the dark cycle), or 6-hours before DID procedures (3-hours before the beginning of the dark cycle). Food deprivation did not significantly alter ethanol drinking or associated blood ethanol concentrations. All values are means + SEM.

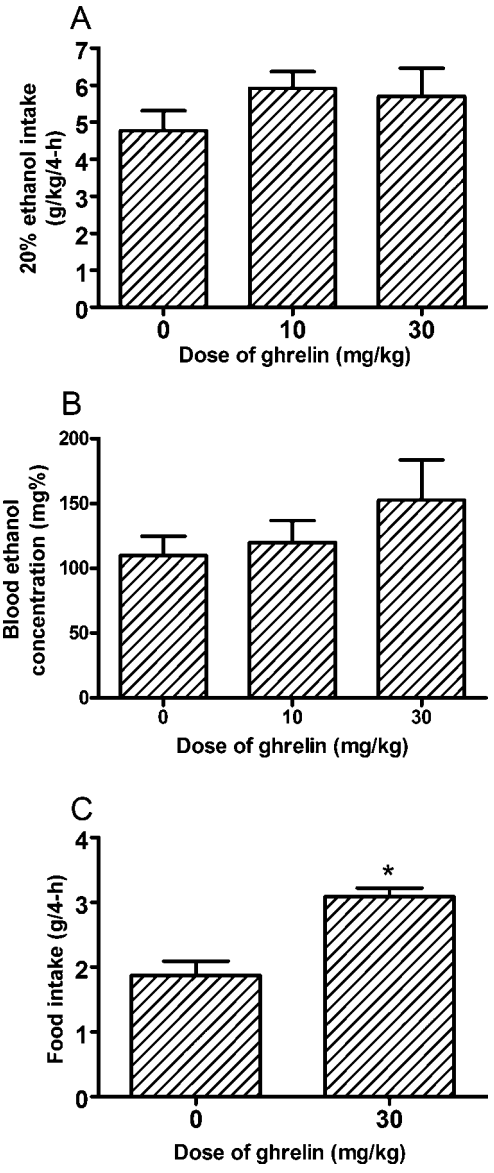

Fig. 2. Consumption of 20% (v/v) ethanol by C57BL/6J mice (**A**) and associated blood ethanol concentrations (**B**) during the 4-hour test day of Experiment 2. Immediately before ethanol access, food was removed from the cages and mice were given an intraperitoneal (i.p.) injection of mouse ghrelin (0, 10, 30 mg/kg). Ghrelin did not significantly alter ethanol consumption or blood ethanol concentrations. Ghrelin (30 mg/kg) did significantly increase 4-hour food intake by C57BL/6J (**C**). All values are means + SEM. *$p < 0.05$.

Experiment 3: Effects of Leptin Injection on Ethanol Intake Associated With DID Procedures and Basal Food Consumption

Ethanol consumption during the 2-hour access on days 1 to 3 of Experiment 3 are presented in the bottom portion of Table 1. ANOVAs revealed no significant group differences in ethanol consumption during days 1 to 3. Ethanol consumption and blood ethanol concentration data from the 4-hour test day of Experiment 3 are presented in Fig. 3*A* and *B*, respectively. One-way ANOVAs performed on these data revealed that the 20 μg/g dose of leptin did not significantly alter ethanol consumption [$F(1, 18) = 0.45$, $p = 0.51$] or blood ethanol levels [$F(1, 18) = 0.46$, $p = 0.50$]. There was a significant correlation between the amount of ethanol consumed on day 4 and blood ethanol concentrations [$R = 0.55$, $p = 0.01$]. The effects of leptin on food consumption are presented in Fig. 3*C*. Importantly, administration of 20 μg/g dose of leptin significantly decreased 4-hour food consumption [$F(1, 8) = 107.88$, $p < 0.001$], verifying that this dose of leptin was physiologically active in C57BL/6J mice.

Experiment 4: Hourly Measures of Food Intake and Saccharin Consumption

The hourly consumption measures for food and 0.05% saccharin from experiment 4 are presented in Fig. 4*A* and *B*, respectively. Visual inspection of the data reveal that C57BL/6J mice showed highest levels of food intake during the period of the dark cycle during which ethanol is administered with DID procedures (Fig. 4*A*). Similarly, 0.05% saccharin solution consumption reached its peak levels during the time period used with DID procedures (Fig. 4*B*).

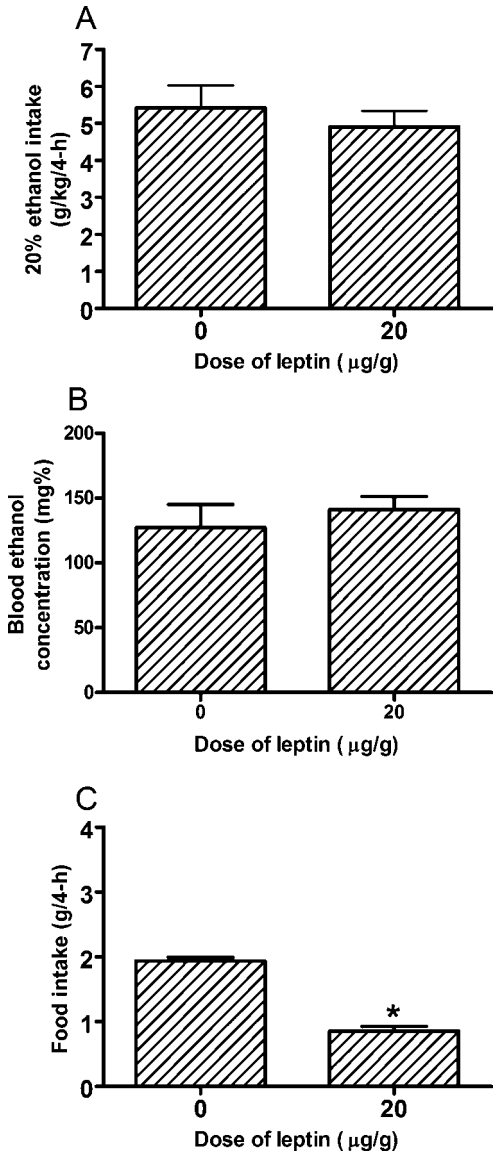

Fig. 3. Consumption of 20% (v/v) ethanol by C57BL/6J mice (**A**) and associated blood ethanol concentrations (**B**) during the 4-hour test day of Experiment 3. Immediately before lights out, food was removed from the cages and mice were given an i.p. injection of mouse leptin (0, 20 μg/g). Leptin did not alter ethanol consumption or blood ethanol concentrations. A 20 μg/g dose of leptin did significantly reduced 4-hour food intake (**C**). All values are means + SEM. *$p < 0.05$.

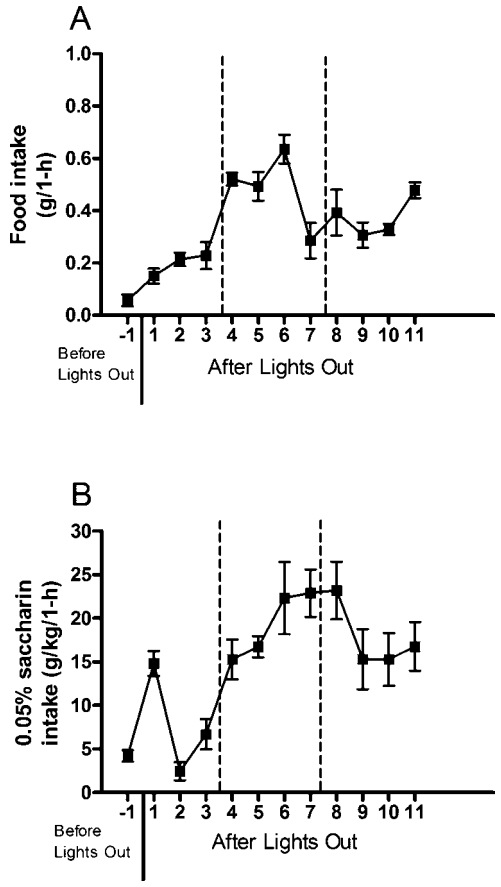

Fig. 4. Hourly consumption of food (**A**) and 0.05% saccharin (**B**) from Experiment 4. Consumption of food and saccharin solution were measured hourly for a 12-hour period from 4-hours before to 4-hours after the 4-hour timeframe that mice received ethanol during DID procedures to determine normal consummatory patterns with a caloric (food) and noncaloric (saccharin) substance. The dotted lines indicate the 4-hour period of time that 20% ethanol is given to mice on the DID test day. All values are means ± SEM.

To analyze these data, we averaged the data into 3, 4-hour blocks of time which corresponded to the 4-hour period before the time of DID procedures, the 4-hour period during the time of DID procedures, and the 4-hour period after the time of DID procedures. A repeated measures ANOVA performed on food intake data revealed a significant effect of time block [$F(2, 12) = 43.92$, $p < 0.001$]. Post hoc tests showed that food intake was significantly higher during the block of time corresponding to DID manipulations relative to either the 4-hour block of time before or after this period. Similarly, a repeated measures ANOVA performed on 0.05% saccharin solution data revealed a significant effect of time block [$F(2, 12) = 53.49$, $p < 0.001$]. Post hoc tests showed that, relative to the 4-hour block of time preceding the period of time used with DID procedures, mice drank significantly more saccharin solution during the 2 other blocks of time.

DISCUSSION

Because DID procedures involve providing C57BL/6J mice with limited access to ethanol during the time of day in which food intake is at its highest levels, it was possible that the high levels of ethanol drinking that are stimulated by DID procedures result from increased caloric need (that is, mice treat ethanol as another calorie-rich food source). If this caloric need hypothesis is correct, 3 predictions should be satisfied: 1) Removal of food should further augment ethanol drinking during DID procedures since ethanol becomes the sole source of calories, 2) pretreatment with an orexigenic peptide (i.e., a peptide that stimulates food intake) should further augment ethanol intake during DID procedures, and 3) pretreatment with an anorectic protein (i.e., an

agent that reduces feeding) should attenuate ethanol intake during DID procedures. Contrary to the caloric need hypothesis, none of these predictions were confirmed. Thus, varying the amount of food deprivation time did not significantly alter ethanol drinking or blood ethanol levels. Furthermore, i.p. injection of ghrelin, a peptide that stimulates food intake (Chen et al., 2004; Wang et al., 2002), failed to increase ethanol drinking in a dose that increased food intake in C57BL/6J mice. Similarly, i.p. injection of leptin, a protein that attenuates feeding (Halaas et al., 1995; Prpic et al., 2003), failed to decrease ethanol intake in a dose that reduced feeding in C57BL/6J mice. Taken together, these observations suggest that the high levels of ethanol drinking promoted by DID procedures are unlikely motivated by caloric need, but rather by other factors such as the pharmacological postingestive effects of ethanol.

Interestingly, during the period of time that C57BL/6J mice received ethanol with DID procedures, they exhibited their highest levels of food intake when measures were collected from 4-hours before to 4-hours after the period of time used with DID procedure (Fig. 4*A*). At first glance, this observation appears to be consistent with the hypothesis that high levels of ethanol intake might be related to caloric need. However, we also observed that C57BL/6J mice showed high levels of saccharin solution consumption during this same period of time (Fig. 4*B*). Since saccharin is a noncaloric compound with reinforcing properties, increased consumption of saccharin cannot be related to increased caloric need. An alternative explanation is that C57BL/6J mice exhibit an increased motivation to consume reinforcing stimuli (such as food, sweet flavors, and ethanol) during the time of day that DID procedures are performed, and increased motivation to consume these reinforcing stimuli is independent of the reinforcer's caloric content.

The present observation that ghrelin did not alter ethanol consumption in C57BL/6J mice is consistent with a recent report showing that hypothalamic infusion of ghrelin increased food intake but did not alter ethanol drinking in Sprague-Dawley rats (Schneider et al., 2007). Interestingly, plasma ghrelin levels were found to be elevated in alcoholics relative to normal individuals (Kraus et al., 2005) and plasma ghrelin levels were positively correlated with self-reports of craving in alcoholics (Addolorato et al., 2006). These observations suggest that while ghrelin may modulate neurobiological pathways involved in craving, this peptide may not directly modulate the ingestion of ethanol. Similarly, the present work with leptin is consistent with the observation that repeated daily injections of leptin failed to alter ethanol drinking in rats, although leptin did appear to augment deprivation-induced increases of ethanol drinking (Kiefer et al., 2001). However, disruption of leptin signaling in mutant mice was associated with reduced ethanol intake (Blednov et al., 2004). Circulating levels of leptin have also been found to be elevated in alcoholics, and are correlated with subjective reports of craving in alcoholics (Kiefer et al., 2005; Nicolas et al., 2001). Thus, as with ghrelin, while leptin may be involved with craving in human alcoholics, its role in modulating ethanol consumption in animal models remains unclear.

In the present report, we observed BECs in C57BL/6J mice that ranged from approximately 100 to 150 mg%. These levels of BECs are consistent with previous reports that have used DID procedures (Kamdar et al., 2007; Rhodes et al., 2005, 2007). Because C57BL/6J mice exhibit signs of behavioral intoxication with BECs in this range (Rhodes et al., 2007), DID procedures appear to provide a valid animal model of drinking to the point of physiological intoxication, and may be useful for identifying targets that may be protective against binge-like ethanol drinking (Kamdar et al., 2007; Moore et al., 2007; Sparta et al., 2008). The present results further strengthen the usefulness of this model by showing that elevated drinking with DID procedures is unlikely related to caloric need.

In conclusion, data obtained in the present study are inconsistent with the hypothesis that C57BL/6J mice consume large amounts of ethanol during DID procedures in order to satisfy a caloric need. Neither food deprivation nor administration of orexigenic or anorectic compounds significantly alter ethanol drinking with DID procedures. A more likely explanation is that increased ethanol drinking is motivated by other factors associated with the pharmacological postingestive effects of ethanol.

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Decreased Immunoreactivity of the Melanocortin Neuropeptide α-Melanocyte-Stimulating Hormone (α-MSH) After Chronic Ethanol Exposure in Sprague–Dawley Rats

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Background: The melanocortin (MC) system is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Recent pharmacologic and genetic evidence suggests that MC receptor (MCR) signaling modulates neurobiologic responses to ethanol and ethanol intake. Because ethanol decreases POMC mRNA levels, we determined if exposure to an ethanol-containing diet (ED) would significantly reduce central immunoreactivity of the MC peptide α-MSH in rats. We also determined if ethanol exposure would alter the immunoreactivity of agouti-related protein (AgRP), an endogenous MCR antagonist.

Methods: Male Sprague–Dawley rats were given 18 days of access to normal rodent chow or a control diet (CD), or short-term (4 days) or long-term (18 days) access to an ED. At the end of the study, rats were perfused with 4% paraformaldehyde and their brains were sectioned into two sets for processing with α-MSH or AgRP immunohistochemistry.

Results: Rats exposed to an ED showed significant reductions of central α-MSH immunoreactivity relative to rats exposed to a control diet (CD) or normal rodent chow. Ethanol-induced reductions of α-MSH immunoreactivity were site-specific and were noted in regions of the hypothalamus and extended amygdala, as well as the paraventricular nucleus of the thalamus. Because there were no differences in body weights or caloric intake between the CD and ED groups, reductions of α-MSH immunoreactivity in ED-treated rats are best explained by ethanol exposure rather than altered energy balance. No significant ethanol-induced alterations in hypothalamic AgRP immunoreactivity were detected.

Conclusions: The present study shows that ethanol site specifically reduces α-MSH immunoreactivity in rat brain. These observations, in tandem with recent pharmacologic and genetic studies, suggest that the endogenous MC system modulates neurobiologic responses to ethanol. Thus, compounds which target MCRs may prove to have therapeutic value in the treatment of excessive ethanol consumption and/or the symptoms associated with ethanol withdrawal.

Key Words: AgRP, α-MSH, Ethanol Consumption, Rats, Melanocortin, POMC.

THE MELANOCORTIN MC SYSTEM is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Central MC peptides are produced by neurons within the hypothalamic arcuate nucleus and the medulla (Dores et al., 1986; Jacobowitz and O'Donohue, 1978; O'Donohue and Dorsa, 1982), and include adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), β-MSH, and γ-MSH (Hadley and Haskell-Luevano, 1999). Because of lack of a critical dibasic site, β-MSH is not processed in rodent brain (Pritchard et al., 2002). Agouti related-protein (AgRP), a neuropeptide produced in the hypothalamus and co-secreted with neuropeptide Y (NPY) in the same synaptic complexes as α-MSH, functions as a natural MC receptor (MCR) antagonist (Shutter et al., 1997).

There are several observations, which suggest that the MC system is a prime candidate for regulating neurobiologic responses to drugs of abuse and drug self-administration. For example, α-MSH administered into the ventral tegmental area (VTA) increases dopamine and DOPAC levels in the nucleus accumbens (NAc; Lindblom et al., 2001), and chronic central infusion of the non-selective MCR agonist, melanotan II

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(MTII), increases dopamine D1 receptor binding in the NAc and dopamine D2 receptor binding in the VTA (Lindblom et al., 2002a). Thus, α-MSH and MTII alter dopamine signaling in these regions. Chronic treatment of a high dose of morphine decreases MC-4 receptor (MC4R) mRNA in the NAc, the periaqueductal gray, and neostriatum (Alvaro et al., 1996), brain regions that modulate drug reward, opiate tolerance, and psychomotor stimulation, respectively (Kalivas and Stewart, 1991; Koob and Bloom, 1988; Wise and Bozarth, 1987). On the other hand, chronic treatment with low doses of morphine or cocaine increases MC4R receptor mRNA in the striatum and NAc (Hsu et al., 2005). Consistent with a role in drug self-administration, central infusion of an MCR agonist decreases the acquisition of heroin self-administration in rats (van Ree et al., 1981).

Importantly, there is also accumulating evidence that MC neuropeptides modulate neurobiologic responses to ethanol. First, α-MSH is expressed in brain regions implicated in ethanol's effects, including the striatum, NAc, VTA, amygdala, hippocampus, and hypothalamus (Bloch et al., 1979; Dube et al., 1978; Jacobowitz and O'Donohue, 1978; O'Donohue and Jacobowitz, 1980; O'Donohue et al., 1979; Yamazoe et al., 1984). Second, rats selectively bred for high ethanol drinking (AA (Alko, Alcohol)) have low levels of MC-3 receptor (MC3R) in the shell of the NAc, but have high levels of MC3R and MC4R in various regions of the hypothalamus, when compared with low ethanol drinking rats (Lindblom et al., 2002b). Third, central infusion of MTII significantly reduced voluntary ethanol drinking in AA rats with an established history of ethanol intake (Ploj et al., 2002). Similarly, MTII-induced reduction of ethanol consumption was shown to be receptor-mediated and not associated with alterations of ethanol metabolism in C57BL/6J mice (Navarro et al., 2003). More recently, ventricular infusion of a selective MC4R agonist significantly reduced ethanol drinking, while ventricular infusion of the non-selective MCR antagonist AgRP-(83–132) significantly increased ethanol drinking, by C57BL/6J mice (Navarro et al., 2005).

In light of the above observations, and the fact that ethanol has direct effects on central POMC mRNA activity (Rasmussen et al., 1998, 2002; Scanlon et al., 1992a; Zhou et al., 2000), an important question is whether ethanol exposure influences central MC neuropeptide content and in which brain regions. To this end, the present study determined if short-term (4 day) and/or long-term (18 day) exposure to an ethanol-containing diet would alter the immunoreactivity of α-MSH in rat brain. Because AgRP is an endogenous MCR antagonist (Shutter et al., 1997), and central infusion of AgRP increases ethanol drinking (Navarro et al., 2005), the immunoreactivity of AgRP in rats following chronic exposure to ethanol was also assessed. Here we show that exposure to an ethanol diet for 4 or 18 days significantly reduced α-MSH immunoreactivity in specific regions of the hypothalamus, thalamus, and extended amygdala while having no effect on the immunoreactivity of AgRP in the hypothalamus.

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats (Charles River, Raleigh, NC, USA) were obtained at 160–180 g and were maintained at 22°C with a 12:12 light/dark cycle. All rats were individually housed in plastic rat cages with free access to water and standard rodent chow (Teklad, Madison, WI) in the vivarium facilities of the Department of Psychology (University of North Carolina). All procedures used in the present study were in compliance with the National Institute of Health guidelines, and all procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC).

Ethanol and Control Diets

The diet was a lactalbumin/dextrose-based, nutritionally complete diet (Dyets, Inc., Bethlehem, PA). Dextrose calories in the control diet (CD) were equated with ethanol calories in the ethanol diet (ED). Rats were habituated to drinking CD in the absence of rodent chow for 2 days (with the exception of the chow control group described below). During the study, all rats with ED were first habituated with 2 days access to a 4.5% (w/v) ED, followed by access to a 7% (w/v) ED for an additional 2 or 16 days. A modified pair-feeding design was used. To equate the caloric intake between groups, the rats maintained on the CD were given a volume of diet equivalent to the average volume consumed the previous day by the rats maintained on the ED. This diet has been used successful to study withdrawal-induced anxiety-like behavior in rats (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2002, 2004). Rodent chow was removed from each rat cage during diet access and rats had access to a second bottle containing tap water at all times.

Following habituation, rats were distributed to 4 groups matched on body weight (n = 10/group) so that each group had approximately the same average weight at the beginning of the study. To control for potential effects of diet on the immunoreactivity of α-MSH or AgRP, one group of rats was maintained on normal rodent chow (Chow) for the entire study. A second control group received CD in place of rodent chow for the duration of the study. A third group was given CD for 14 days, the 4.5% ED for 2 days, and the 7% ED for 2 days (group ED4). A fourth group received the 4.5% ED for 2 days, followed by the 7% ED for 16 days (group ED18). Rats that experienced a similar protocol (15 days of access to at 7% ED) achieved blood ethanol concentrations ranging from 100 mg/dl after the first day of access to 200 mg/dl during the 15th day of access (Overstreet et al., 2002). Throughout the study, diet intake and body weight measures were recorded daily.

Perfusions, Brain Preparation, and Immunohistochemistry (IHC)

Immunohistochemistry procedures were based on those routinely used in our laboratory (Hayes et al., 2005; Knapp et al., 1998; Thiele et al., 1996, 1997, 1998a,b, 2000). Immediately after 18 days of access to diet, rats were injected with pentobarbital (100 mg/kg) and were then perfused within 10 minutes transcardially with 0.1 mM phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in phosphate buffer. Rats were perfused in pairs, and the order that rats were perfused was counterbalanced by diet condition. Rats had access to diet up to the time of perfusions to avoid ethanol withdrawal in the ED groups. All perfusions were completed within a 5-hour window of time. The brains were collected and post-fixed in paraformaldehyde for 24 hours at 4°C, at which point they were transferred to PBS. Rat brains were cut using a vibratome into 40 μm

sections that were then stored in PBS until the IHC assay. Sections were evenly divided into two sets (every-other section) for processing with α-MSH or AgRP antibodies. After rinsing in fresh PBS 4 times (10 minutes each), tissue sections were blocked in 10% rabbit serum (for α-MSH) or 10% goat serum (for AgRP) and 0.1% triton-X-100 in PBS for 1 hour. Sections were then transferred to fresh PBS containing primary sheep anti-α-MSH (Millipore, Billerica, MA; 1:10,000) or primary rabbit anti-AgRP (Phoenix Pharmaceuticals, Inc., Burlingame, CA; 1:4,000) for 3 days at 4°C. As a control to determine if staining required the presence of the primary antibodies, some sections were run through the assay without primary antibody (α-MSH or AgRP). In each assay described below, tissue processed without the primary antibody failed to show staining that was evident in tissue processed with primary antibody. After the 3 days of incubation, the sections were rinsed 4 times and then processed with Vectastain Elite kits (Vector Labs) as per the manufacturer's instructions for standard ABC/HRP/diaminobenzidine-based immunohistochemistry. The sections processed for α-MSH or AgRP were visualized by reacting the sections with a 3,3′-diamino-benzidine tetrahydrochloride (DAB, Polysciences, Inc., Warrington, PA) reaction solution containing 0.05% DAB, 0.005% cobalt, 0.007% nickel ammonium sulfate, and 0.006% hydrogen peroxide. All sections were mounted on glass slides, air-dried overnight, and cover slipped for viewing.

Digital images of α-MSH and AgRP immunohistochemistry were obtained on a Nikon E400 microscope equipped with a Nikon Digital Sight DS-U1 digital camera run with Nikon-provided software. For analysis, great care was taken to match sections through the same region of brain and at the same level using anatomic landmarks with the aid of a rat stereotaxic atlas (Paxinos and Watson, 1986). For cell counting, all visible cell bodies stained within the defined brain region were counted manually by an experimenter blinded to group condition. Data from each brain region in an animal were calculated by taking the average counts from 2 brain slices. Data from each slice were calculated by taking the average counts from the left and right sides of the brain at the specific brain region of interest. For non-cell body localization of the α-MSH or AgRP in a given brain region, densitometric procedures were used to assess protein levels. Flat-field corrected digital pictures (8-bit grayscale) were taken using the Digital Sight DS-U1 camera and density of staining was analyzed using Image J software (Image J, National Institute of Health, Bethesda, MD) by calculating the percent of the total area examined that showed signal (cell bodies and processes) relative to a subthreshold background. The size of the areas that were analyzed was the same between animals and groups. The subthreshold level for the images was set in such a way that any area without an experimenter defined level of staining was given a value of zero. Anatomically matched pictures of the left and right sides of the brain were used to produce an average density for each brain region from each slice. In all cases, quantification of immunohistochemistry data was conducted by an experimenter that was blinded to group identity.

Data Analyses

All data in this report are presented as mean ± SEM differences between groups were analyzed using one-way analyses of variance (ANOVA) procedures. Because we expected that ethanol-induced alterations of α-MSH would be site specific, separate ANOVAs were performed for each brain region. When significant differences are found, post hoc analyses were conducted using the Tukey's HSD test. For one set of analyses, *t*-tests were used for planned comparisons in accordance with a priori hypotheses. In all cases, $p < 0.05$ (two-tailed) was used as the level of statistical significance.

RESULTS

Body Weights

Because both α-MSH and AgRP have been implicated in the modulation of food intake and body weight (Sainsbury et al., 2002), it was important to determine if there were body weight differences between the different treatment groups over the course of the experiment. A one-way ANOVA performed on baseline body weight data collected the day before the initiation of diet exposure failed to achieve statistical significance ($F_{3,\ 36} = 0.009$; $p > 0.05$), a result verifying the similar body weights between the Chow (286.6 ± 6.5 g), CD (287.7 ± 5.7 g), ED4 (287.7 ± 4.4 g), and ED18 (287.5 ± 4.8 g) groups. A one-way ANOVA performed on body weight data collected at the end of the study was significant ($F_{3,\ 36} = 16.19$; $p < 0.001$). In this case, Tukey's HSD post-hoc tests revealed that while the

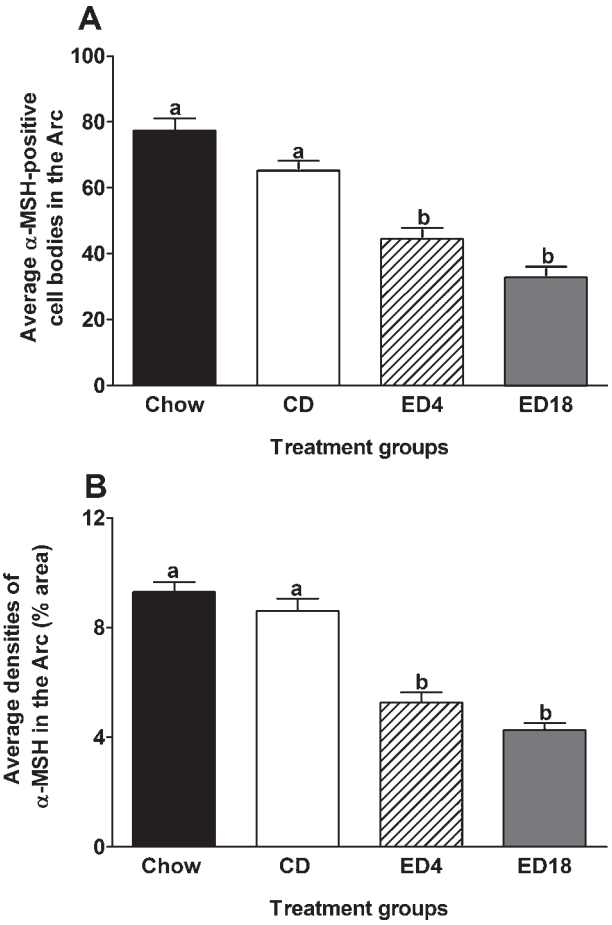

Fig. 1. Quantification of α-melanocyte-stimulating hormone (MSH) immunoreactivity in the arcuate nucleus of the hypothalamus (Arc). Quantification was done by counting α-MSH-positive cell bodies (**A**) or by measuring the density of α-MSH staining (**B**) using Image J software, which calculated the percent of the total area examined (% area) that showed signal (cell bodies and processes) relative to a subthreshold background. Groups were given 18-days of access to normal rodent chow (Chow) or an ethanol-free control diet (CD), or an ethanol diet for 4 (ED4) or 18 (ED18) days. Values are represented as mean ± SEM. There are statistical differences between groups that do not share overlapping lettering (a or b; $p < 0.05$).

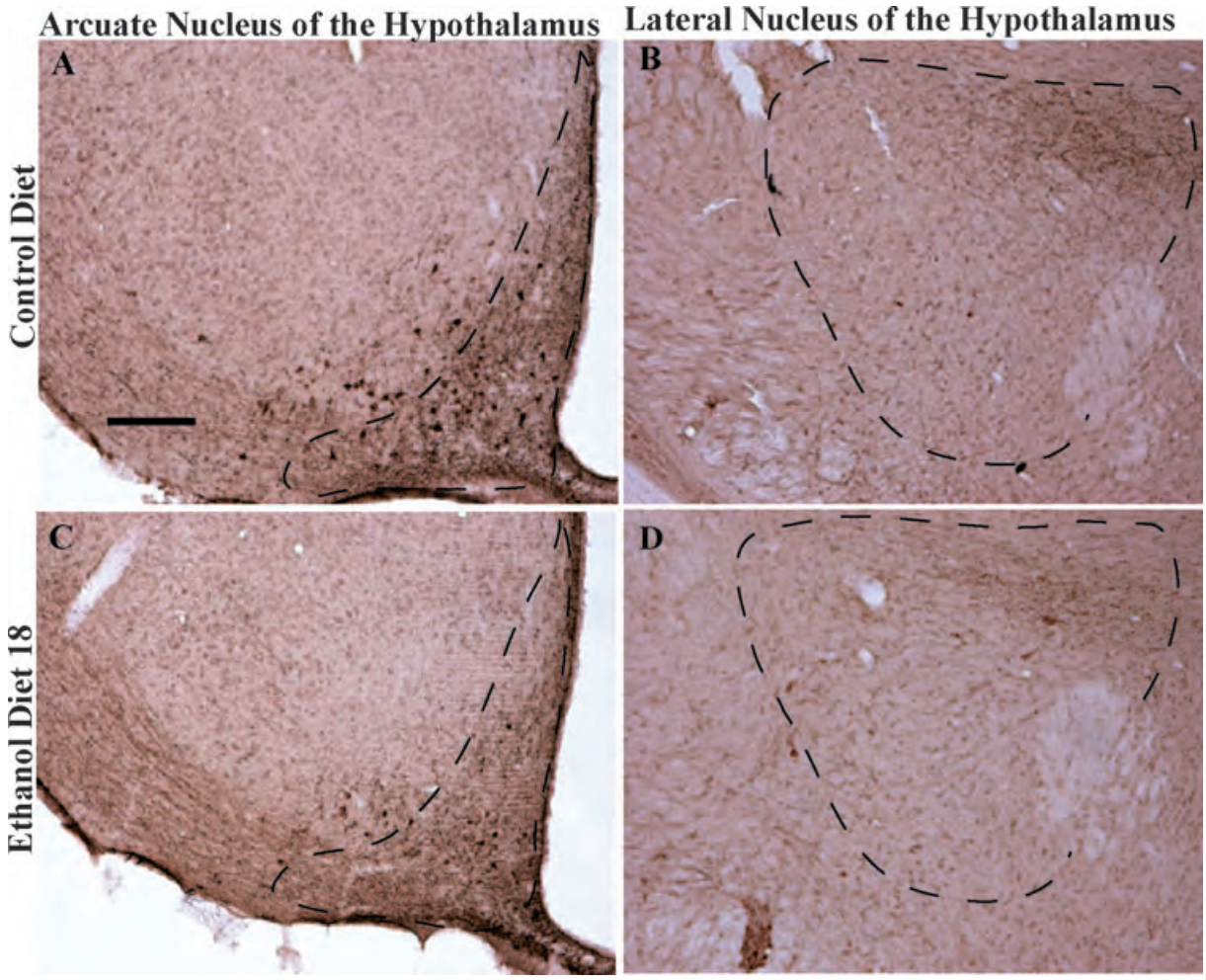

Fig. 2. Representative photomicrographs of 40 μm coronal sections showing α-melanocyte-stimulating hormone (MSH) immunoreactivity through the arcuate nucleus of the hypothalamus (**A** and **C**) and the lateral nucleus of the hypothalamus (**B** and **D**) of rats given 18 days of exposure to the control diet or the ethanol diet (Ethanol Diet 18). Dashed line depicts the region that was selected for quantification. Images were photographed and quantified at a magnification of 10×. Scale bar = 200 μm. α-MSH immunoreactivity in the arcuate nucleus appears in cell bodies, while staining in the lateral hypothalamus is located primarily in cellular processes.

Chow group (396.5 ± 9.4 g) weighed significantly more than the CD (344.8 ± 5.0 g), ED4 (339.9 ± 6.1 g), and ED18 (326.8 ± 9.1 g) groups, none of the diet treated groups differed significantly from one another. Furthermore, as the pair-feeding procedure equated the volume of diet consumption between CD and ED groups, and because the CD and ED were calorically equated, there were no differences in caloric intake between groups given access to liquid diet. These observations reinforce the conclusion the differences between CD- and ED-treated groups below are best explained by the presence or absence of ethanol exposure rather than group differences in caloric intake or body weight.

Immunoreactivity of α-MSH in Regions of the Hypothalamus After Ethanol Exposure

Arcuate Nucleus of the Hypothalamus (Arc). Data representing the average immunoreactivity of α-MSH in the Arc are presented in Fig. 1, and represented photomicrographs of α-MSH immunoreactivity in the Arc of groups CD and ED18 are depicted in Fig. 2*A* and 2*C* (to conserve space, pictures of sections from the Chow and ED4 groups are not presented in photomicrograph figures). The Arc was the only brain region in which α-MSH was expressed in cell bodies rather than cellular processes. Thus, to verify that cell counting and densitometric procedures yielded similar results, we quantified and analyzed α-MSH immunoreactivity in this region using both procedures. Average cell counts of α-MSH-positive cells in the Arc are presented in Fig. 1*A*. A one-way ANOVA performed on these data was significant ($F_{3,\ 36} = 36.28$; $p < 0.001$). Tukey's HSD post hoc tests revealed that both groups ED4 and ED18 showed significantly lower immunoreactivity of α-MSH relative to the control groups (Chow and CD). Average densities (% area) of α-MSH immunoreactivity in the Arc are presented in Fig. 1*B*. Similar to the cell counting data, a one-way ANOVA

performed on density data was significant ($F_{3,\ 36} = 44.81$; $p < 0.001$), and Tukey's HSD tests showed that groups ED4 and ED18 showed significantly lower immunoreactivity of α-MSH relative to groups Chow and CD.

Lateral (LH), Dorsomedial (DMH), and Paraventricular (PVN) Nuclei of the Hypothalamus.

Data representing the average immunoreactivity of α-MSH in the LH, DMH, and PVN are presented in Fig. 3, and representative photomicrographs of α-MSH immunoreactivity in the LH of groups CD and ED18 are depicted in Fig. 2*B* and 2*D*. A one-way ANOVA performed on average densities of α-MSH immunoreactivity in the LH was significant ($F_{3,\ 36} = 5.77$; $p < 0.05$). Tukey's HSD tests revealed that while group ED18 had significantly lower α-MSH immunoreactivity relative to both control groups (Chow and CD), group ED4 did not differ significantly from the control groups (Fig. 3*A*). One-way ANOVAs performed on average densities of α-MSH immunoreactivity in the DMH ($F_{3,\ 36} = 0.38$; $p > 0.05$) and PVN ($F_{3,\ 36} = 0.23$; $p > 0.05$) both failed to achieve statistical significance (see Fig. 3*B* and *C*, respectively).

Immunoreactivity of α-MSH in the Extended Amygdala, Thalamus, and Periaqueductal Gray After Ethanol Exposure

Central Nucleus of the Amygdala (CeA) and Bed Nucleus of the Stria Terminalis (BNST).

Data representing the average immunoreactivity of α-MSH in the extended amygdala regions CeA and BNST are presented in Fig. 4*A* and *B*, respectively, and representative photomicrographs from groups CD and ED18 in these regions are presented in Fig. 5. A one-way ANOVA performed on the average densities of α-MSH immunoreactivity in the CeA was significant ($F_{3,\ 36} = 3.05$; $p < 0.05$). Although Tukey's HSD tests did not reveal significant group differences, planned *t*-test comparisons indicated that while group ED18 showed significantly lower levels of α-MSH immunoreactivity relative to the control groups (Chow and CD), group ED4 did not differ significantly from the control groups. A one-way ANOVA performed on average densities of α-MSH immunoreactivity in the BNST was significant ($F_{3,\ 36} = 17.96$; $p < 0.001$), and Tukey's HSD tests revealed that both of the ethanol diet groups (ED4 and ED18) showed significantly lower levels of α-MSH immunoreactivity relative to the control groups (Chow and CD).

Paraventricular Nucleus of the Thalamus (PVT) and Periaqueductal Gray (PAG).

Data representing the average immunoreactivity of α-MSH in the PVT and PAG are presented in Fig. 4*C* and 4*D*, respectively, and representative photomicrographs from groups CD and ED18 in the PVT are presented in Fig. 6. A one-way ANOVA performed on the average densities of α-MSH immunoreactivity in the PVT was significant ($F_{3,\ 36} = 16.14$; $p < 0.001$) and Tukey's HSD tests showed that both groups ED4 and ED18 had significantly lower α-MSH immunoreactivity relative to the control groups (Chow and CD).

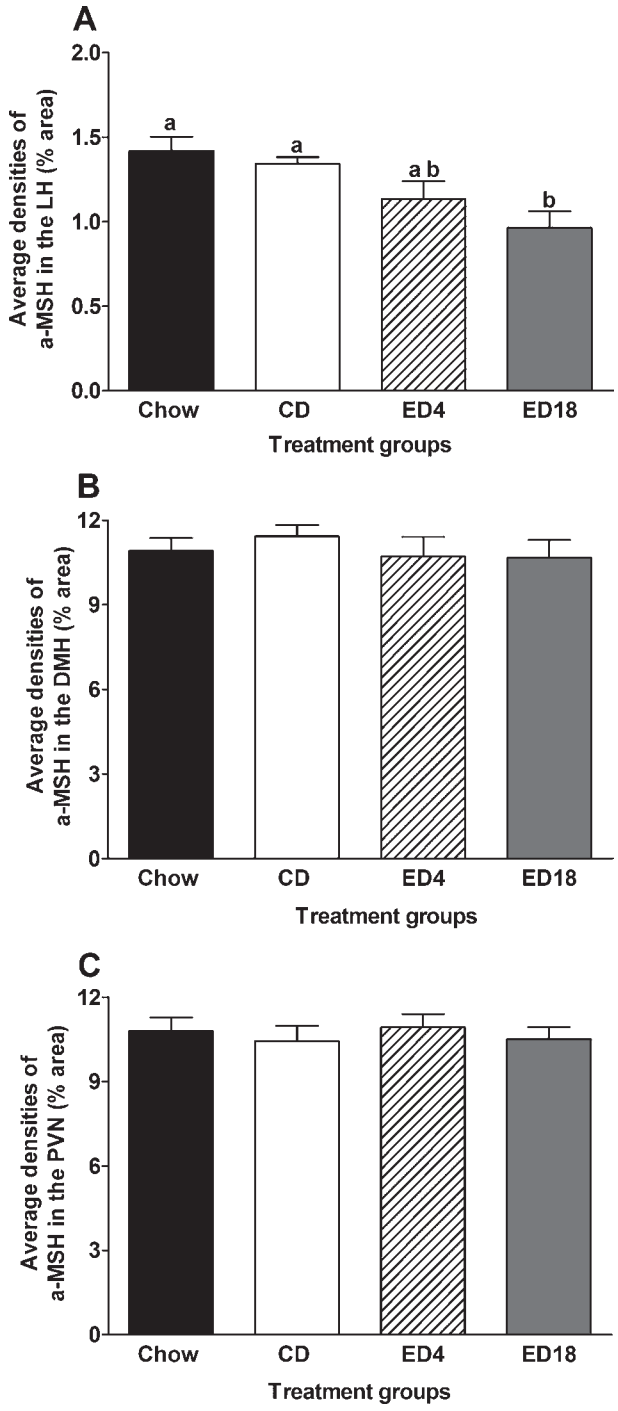

Fig. 3. Quantification of α-melanocyte-stimulating hormone immunoreactivity (% area) in the lateral nucleus of the hypothalamus (LH; **A**), the dorsomedial nucleus of the hypothalamus (DMH; **B**), and the paraventricular nucleus of the hypothalamus (PVN; **C**). Groups were given 18 days of access to normal rodent chow (Chow) or an ethanol-free control diet (CD), or an ethanol diet for 4 (ED4) or 18 (ED18) days. Values are represented as mean ± SEM. There are statistical differences between groups that do not share overlapping lettering (a or b; $p < 0.05$).

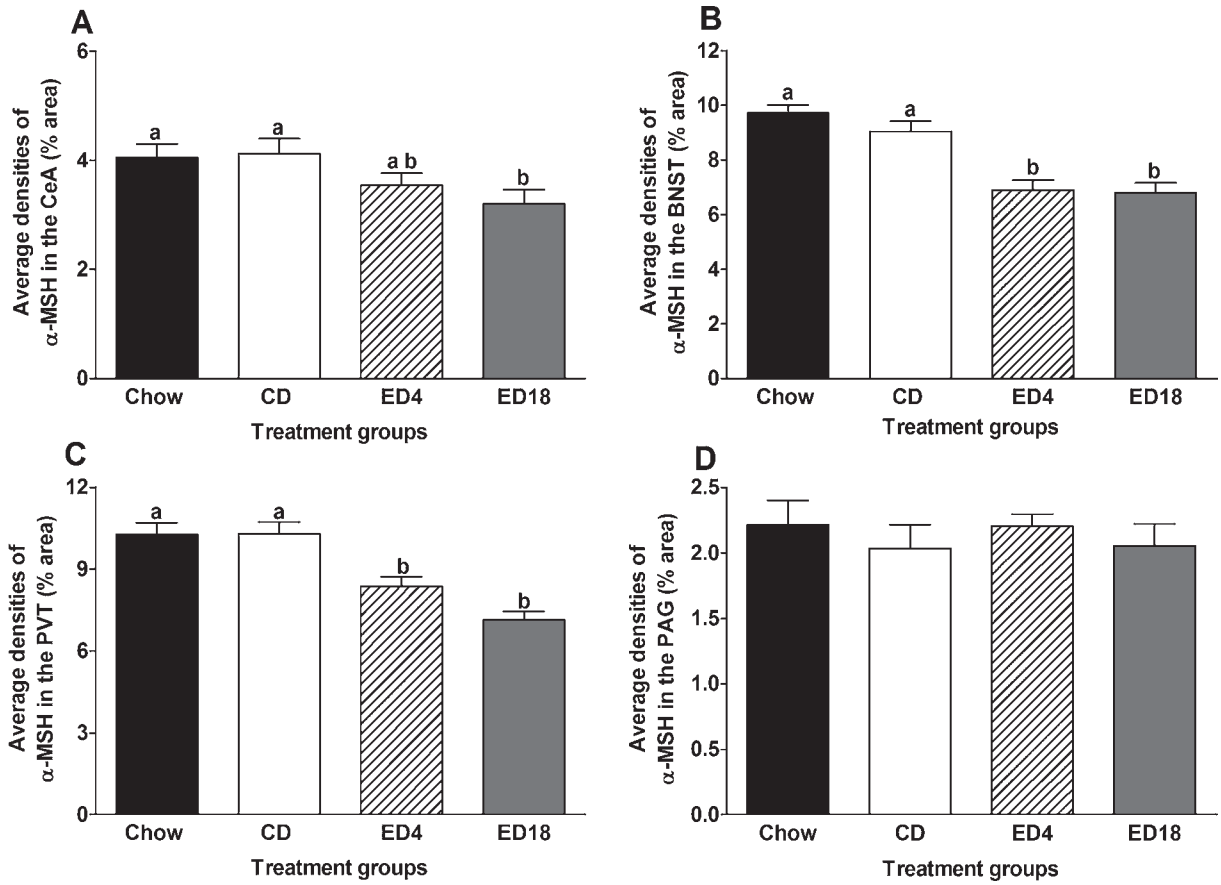

Fig. 4. Quantification of α-melanocyte-stimulating hormone immunoreactivity (% area) in the central nucleus of the amygdala (CeA; **A**), the bed nucleus of the stria terminalis (BNST; **B**), the paraventricular nucleus of the thalamus (PVT; **C**), and the periaqueductal gray (PAG; **D**). Groups were given 18 days of access to normal rodent chow (Chow) or an ethanol-free control diet (CD), or an ethanol diet for 4 (ED4) or 18 (ED18) days. Values are represented as mean ± SEM. There are statistical differences between groups that do not share overlapping lettering (a or b; $p < 0.05$).

A one-way ANOVA performed on the average densities of α-MSH immunoreactivity in the PAG failed to reach statistical significance ($F_{3, 36} = 0.32$; $p > 0.05$).

Immunoreactivity of AgRP in the Arc After Ethanol Exposure

Data representing the average densities (% of area) of AgRP immunoreactivity in the Arc are presented in Fig. 7, and representative photomicrographs from groups CD and ED18 in this region are depicted in Fig. 8. A one-way ANOVA performed on these data failed to achieve statistical significance ($F_{3, 36} = 1.20$; $p > 0.05$).

DISCUSSION

Here we show that Sprague–Dawley rats exposed to an ethanol containing diet exhibit significant reductions of central α-MSH immunoreactivity relative to rats exposed to a control diet or normal rodent chow. Ethanol-induced reductions of α-MSH immunoreactivity were noted in regions of the hypothalamus (the Arc and LH) and extended amygdala (the CeA and BNST) as well as the paraventricular nucleus of the thalamus (PVT). Regions that did not show ethanol-induced alterations of α-MSH immunoreactivity were the DMH, PVN, and PAG. We did not find quantifiable levels of α-MSH immunoreactivity in other brain regions. The observation that ethanol exposure reduced α-MSH in some, but not all, brain regions indicates that the effects of ethanol exposure on α-MSH immunoreactivity are brain-region specific. This observation limits the likelihood that ethanol-induced reductions of α-MSH immunoreactivity were secondary to any global effects of ethanol on brain morphology or cellular toxicity. Importantly, because there were no differences at the end of the study in body weights between the group that received the CD versus the groups that received ED, and because caloric intake between diet groups were matched, reductions of α-MSH immunoreactivity in ED-treated groups are best explained by ethanol exposure, rather than group differences in caloric intake or body weight. As rats that experienced a similar protocol (15-days of access to at 7% ED) achieved blood ethanol concentrations ranging from 100 mg/dl after the first day of access to 200 mg/dl during

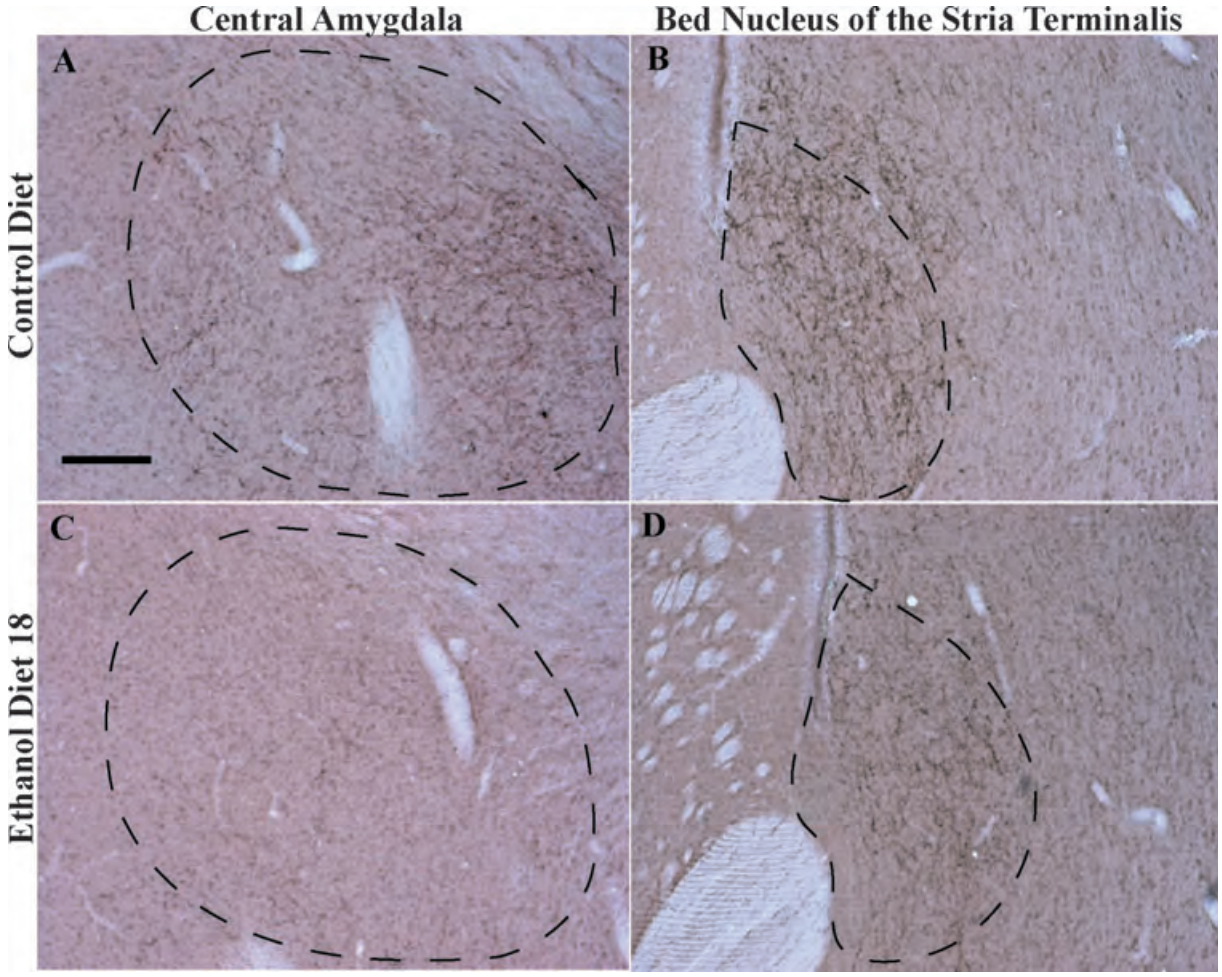

Fig. 5. Representative photomicrographs of 40 μm coronal sections showing α-melanocyte-stimulating hormone (MSH) immunoreactivity through the central nucleus of the amygdala (**A** and **C**) and the bed nucleus of the stria terminalis (**B** and **D**) of rats given 18 days of exposure to the control diet or the ethanol diet (Ethanol Diet 18). Dashed line depicts the region that was selected for quantification. Images were photographed and quantified at a magnification of 10×. Scale bar = 200 μm. α-MSH immunoreactivity in these regions is located primarily in cellular processes.

the 15th day of access (Overstreet et al., 2002), the effects of ethanol exposure on α-MSH immunoreactivity are probably related to the central pharmacologic actions of this drug. These observations extend an earlier finding of ED-induced reduction of α-MSH immunoreactivity in the Arc and substantia nigra (Rainero et al., 1990). On the other hand, neither 4 or 18 days of exposure to ED caused significant alterations of AgRP immunoreactivity in the Arc. Given that we observed AgRP immunoreactivity in only one brain region, and in the absence of other measures (e.g., mRNA levels), it would be premature to conclude that ethanol exposure does not influence AgRP immunoreactivity.

With the exception of the LH and the CeA, which showed reductions of α-MSH immunoreactivity only after 18 days of exposure to the ED, all other regions that were affected by ED had reduced α-MSH immunoreactivity with both short-term (4 days) and long-term (18 days) exposure to ethanol. This observation raises the interesting possibility that the reduction of α-MSH immunoreactivity in the LH and CeA progresses with the development of ethanol dependence over the course of continued ethanol exposure. Consistent with this idea, Sprague–Dawley rats show increased withdrawal-induced anxiety-like behavior after 17-days of access to a 7% ED (Knapp et al., 2004), but not after 5-days of access to a 7% ED (Overstreet et al., 2005). On the other hand, reductions of α-MSH immunoreactivity in regions after only 4-days of ED access may represent neurobiologic responses to ethanol prior to the development of ethanol dependence.

With immunohistochemistry procedures, reduced α-MSH immunoreactivity in response to ethanol exposure could indicate that ethanol inhibits normal α-MSH signaling via reduced production of α-MSH and/or interference of the normal transport of α-MSH to the terminals. Alternatively, ethanol-induced reduction of α-MSH immunoreactivity may reflect an augmentation of α-MSH signaling via potentiated release and/or the inhibition of α-MSH re-uptake into presynaptic terminals. While either option is possible, the observation that exposure to ethanol inhibits

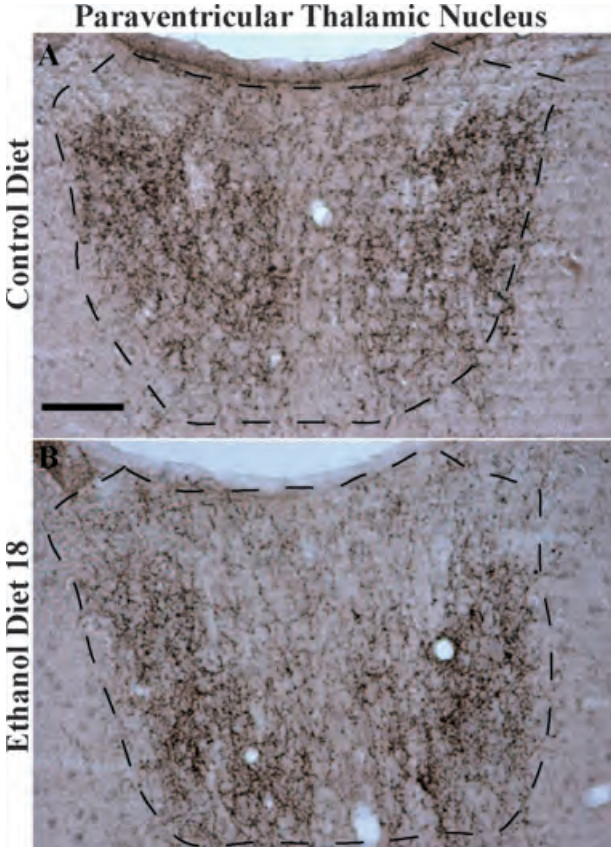

Fig. 6. Representative photomicrographs of 40 μm coronal sections showing α-melanocyte-stimulating hormone (MSH) immunoreactivity through the paraventricular nucleus of the thalamus of rats given 18 days of exposure to the control diet (**A**) or the ethanol diet (**B**, Ethanol Diet 18). Dashed line depicts the region that was selected for quantification. Images were photographed and quantified at a magnification of 10×. Scale bar = 200 μm. α-MSH immunoreactivity in this region is located primarily in cellular processes.

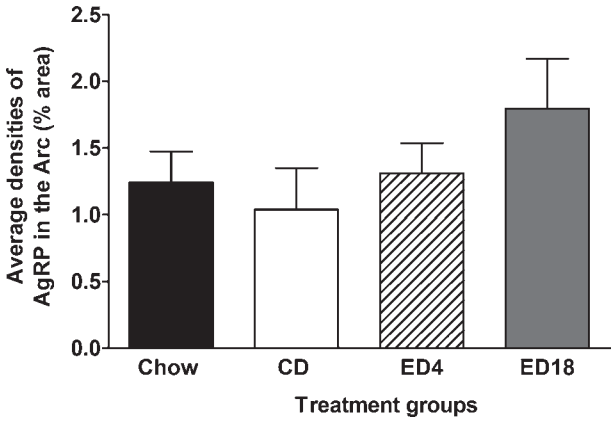

Fig. 7. Quantification of AgRP immunoreactivity (% area) in arcuate nucleus of the hypothalamus (Arc). Groups were given 18 days of access to normal rodent chow (Chow) or an ethanol-free control diet (CD), or an ethanol diet for 4 (ED4) or 18 (ED18) days. Values are represented as mean ± SEM.

POMC mRNA (Rasmussen et al., 2002; Scanlon et al., 1992b; Zhou et al., 2000) leads us to speculate that chronic exposure to ethanol disrupts normal α-MSH synthesis which ultimately depletes α-MSH immunoreactivity in the terminals of brain regions involved with neurobiologic responses to ethanol. A mechanism for the effects of ethanol on POMC mRNA may involve GABA signaling. Ethanol is a sedative drug that enhances GABAergic transmission (Criswell and Breese, 2005; Weiner and Valenzuela, 2006). Because peripheral administration of GABA agonists reduced POMC mRNA in the arcuate nucleus of Sprague–Dawley rats (Garcia de Yebenes and Pelletier, 1994), it is likely that reduction of POMC mRNA (and thus reduced α-MSH immunoreactivity) results from increased GABAergic transmission in the presence of ethanol.

Central MC signaling modulates food intake and body weight (Shimizu et al., 2007). Site-directed injection of a melanocortin receptor agonist into the PVN, DMH, LH, Arc, and CeA have been shown to significantly reduce food intake in rats (Giraudo et al., 1998; Kim et al., 2000), and ventricular infusion of the melanocortin receptor agonist MTII, significantly elevated c-Fos immunoreactivity in the PVN and Arc (Thiele et al., 1998b). While there is clear overlap of brain regions in which melanocortin signaling controls feeding with those regions exhibiting ethanol-induced reductions of α-MSH immunoreactivity, the PVN and DMH are critical sites in which α-MSH signaling modulates feeding, yet no effects of ethanol exposure on α-MSH immunoreactivity in the PVN or DMH were noted in the present report. This observation, and the fact that ethanol-induced reductions of α-MSH immunoreactivity were not attributable to calories or body weight change, suggests that the α-MSH pathways that modulate feeding/body weight and neurobiologic responses to ethanol are not identical.

It is of interest to consider the possible role(s) that α-MSH signaling plays in the modulation of neurobiologic responses to ethanol. One possibility is that α-MSH signaling is part of a mechanism that prevents excessive ethanol drinking. While we did not assess the effects of ED exposure on voluntary ethanol drinking in the present study, there is abundant evidence that chronic exposure to an ethanol-containing diet or ethanol vapor augments voluntary ethanol drinking during periods of withdrawal (Becker and Lopez, 2004; Finn et al., 2007; Schulteis et al., 1996; Valdez et al., 2002). Thus, down-regulation of α-MSH signaling following chronic exposure to ethanol may leave rats vulnerable to excessive ethanol intake. Consistent with this hypothesis are the observations that MCR agonists attenuate ethanol drinking in rats and mice (Navarro et al., 2003, 2005; Ploj et al., 2002), while blockade of the MCR augments ethanol drinking in mice (Navarro et al., 2005). As pretreatment with MTII blocked ethanol-induced decreases of Met-enkephalin-Arg^{6}Phe^7 (MEAP) immunoreactivity in the ventral tegmental area of rats, it is possible that MCR agonism reduces ethanol intake through actions on the opioid system (Ploj et al., 2002). More recently we found that site-directed infusion of a selective MC4R

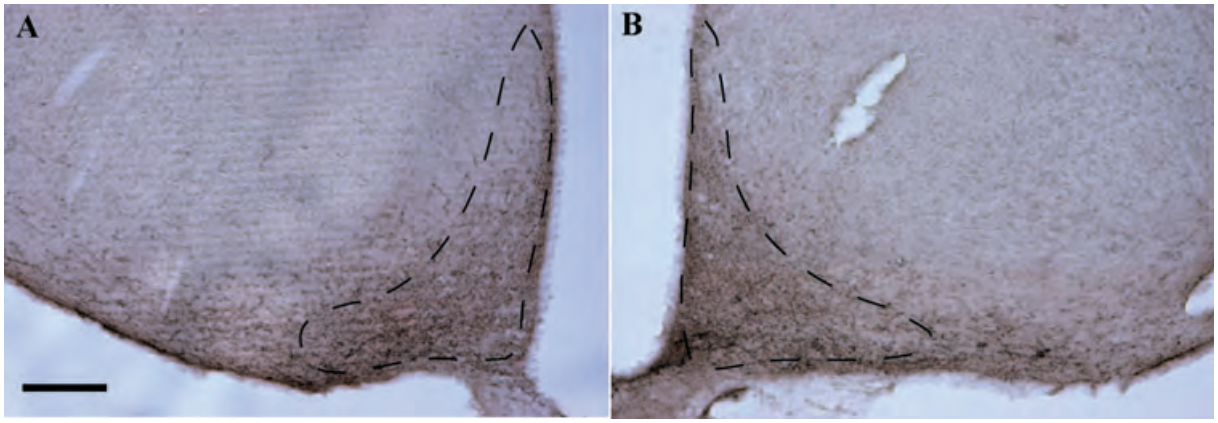

Fig. 8. Representative photomicrographs of 40 μm coronal sections showing AgRP immunoreactivity through the arcuate nucleus of the hypothalamus of rats given 18 days of exposure ethanol diet (Ethanol Diet 18; **A**) or to control diet (**B**). Dashed line depicts the region that was selected for quantification. Images were photographed and quantified at a magnification of 10×. Scale bar = 200 μm. AgRP immunoreactivity in this region is located primarily in cellular processes.

antagonist into the NAc increased ethanol drinking in Sprague–Dawley rats (Carvajal et al., 2007), a finding that suggests that endogenous α-MSH may negatively modulate the rewarding properties of ethanol via MC4R signaling in the NAc. It should be noted, however, that there is also evidence that MCR signaling enhances the rewarding properties of cocaine and amphetamine (Cabeza de Vaca et al., 2002; Hsu et al., 2005). Regardless, while the mechanisms by which MCR signaling modulates the consumption of ethanol and other drugs of abuse are not completely understood, such mechanisms may involve an interaction of α-MSH with the opioid system as mentioned above (Ploj et al., 2002), and/or by α-MSH actions within the mesolimbic dopaminergic pathway (Lindblom et al., 2001, 2002a).

Another possibility is the reduced α-MSH immunoreactivity following ethanol exposure contributes to the anxiolytic effects of ethanol. In fact, there is a growing body of literature showing that MCR agonists induce, while MCR antagonists inhibit, anxiety-like behaviors in rodents (Chaki and Okuyama, 2005; Chaki et al., 2003, 2005; Kokare et al., 2005; Nozawa et al., 2007; Shimazaki and Chaki, 2005). Consistent with this idea is a recent report showing that the anxiolytic effect of an intraperitoneal ethanol injection was suppressed by central infusion of α-MSH but enhanced by central infusion of a MC4R antagonist or antiserum against α-MSH (Kokare et al., 2006). Thus, ethanol-induced reduction of α-MSH immunoreactivity may be part of the mechanism by which ethanol induces anxiolytic effects. If in fact ethanol-induced reduction of α-MSH immunoreactivity contributes to the anxiolytic effects of ethanol, one would predict that acute ethanol exposure would induce rapid reductions of α-MSH immunoreactivity in critical brain regions as the anxiolytic effects of ethanol are immediate. Furthermore, α-MSH immunoreactivity would be expected to return to normal levels soon after ethanol is eliminated from the blood. These are important questions that will be the focus of future research. Taken together, MCR signaling may modulate any number of neurobiologic responses to ethanol, including ethanol ingestion, the rewarding properties of ethanol, and/or ethanol's anxiolytic properties. It will be important to determine the specific brain regions in which α-MSH modulates these different neurobiologic responses.

In conclusion, here we show that chronic exposure to an ethanol-containing diet leads to significant reductions of α-MSH within specific brain regions. Reductions of α-MSH are not related to group differences in body weight or caloric intake. Future research is needed to determine the precise mechanism by which ethanol modulates central α-MSH immunoreactivity. The present observations, in tandem with recent genetic and pharmacologic studies, strongly suggest that the endogenous MC system modulates neurobiologic responses to ethanol. Thus, compounds which target MCRs may prove to have therapeutic value in the treatment of excessive ethanol consumption and/or the symptoms associated with ethanol withdrawal.

ACKNOWLEDGMENTS

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The Alcohol Deprivation Effect in C57BL/6J Mice is Observed Using Operant Self-Administration Procedures and is Modulated by CRF-1 Receptor Signaling

Dennis R. Sparta, Frank M. Ferraro III, Jon R. Fee, Darin J. Knapp, George R. Breese, and Todd E. Thiele

Background: The alcohol deprivation effect (ADE) is characterized by transient excessive alcohol consumption upon reinstatement of ethanol following a period of ethanol deprivation. While this phenomenon has been observed in rats using both bottle drinking (consummatory behavior) and operant self-administration (consummatory and appetitive "ethanol-seeking" behavior) procedures, ADE studies in mice have primarily relied on bottle drinking measures. Furthermore, the neurochemical pathways that modulate the ADE are not well understood. Therefore, we determined whether the ADE can be observed in C57BL/6J mice using operant self-administration procedures and if expression of the ADE is modulated by the corticotropin releasing factor-1 (CRF-1) receptor.

Methods: C57BL/6J mice were trained in a 2-hour operant self-administration paradigm to lever press for 10% ethanol or water on separate response keys. Between operant sessions, mice had access to ethanol in their homecage. Once stable responding occurred, mice were deprived of ethanol for 4 days and were then retested with ethanol in the operant paradigm for 3 consecutive days. Next, to assess the role of the CRF-1 receptor, mice were given intraperitoneal (i.p.) injection (0, 10, or 20 mg/kg) of the CRF-1 receptor antagonist CP-154,526 30 minutes before ADE testing. Additional experiments assessed (i) ADE responding in which the alternate response lever was inactive, (ii) the effects of CP-154,526 on self-administration of a 1% sucrose solution following 4 days of deprivation, and (iii) ADE responding in which mice did not received i.p. injections throughout the experiment.

Results: Mice exhibited a significant increase in postdeprivation lever responding for ethanol with either a water reinforced or inactive alternate lever. Interestingly, i.p. injection of a 10 mg/kg dose of CP-154,526 protected against the ADE while not affecting lever responding for a sucrose solution. Finally, baseline and deprivation-induced increases of ethanol reinforced lever responding were greater in mice not given i.p. injections.

Conclusions: The ADE in C57BL/6J mice can be modeled using the operant self-administration paradigm and increased ethanol self-administration associated with the ADE is modulated by CRF-1 receptor signaling.

Key Words: C57BL/6J Mice, Alcohol Deprivation Effect, Two-Bottle Consumption, Operant Self-Administration, Corticotropin Releasing Factor.

ALCOHOL RELAPSE IS a major problem in the treatment of alcoholism. Approximately 60% to 80% of abstinent alcoholics will relapse at 1 point in their lifetime (Barrick and Connors, 2002; Chiauzzi, 1991). Thus, understanding the neurobiology of relapse and associated behaviors is a critical step toward the development of drugs aimed at treating alcoholism. Relapse after long periods of abstinence is frequently associated with excessive, or uncontrolled, ethanol drinking (Holter et al., 2000). Recent procedures have been developed and validated as animal models of this uncontrolled ethanol drinking. One procedure involves periodic deprivation from ethanol after which animals consume significantly more ethanol than they had consumed prior to the deprivation period. This phenomenon has been labeled the alcohol deprivation effect (ADE) and is thought to model compulsive uncontrolled relapse drinking characteristic of alcohol-dependent humans (Spanagel and Holter, 1999).

The ADE is a robust phenomenon evident in rats (Backstrom et al., 2004b; Bell et al., 2004; Colombo et al., 2003; Dayas et al., 2004; Fullgrabe et al., 2007;

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Funk et al., 2004; Heyser et al., 1997; Holter et al., 2000; McKinzie et al., 1998; Oster et al., 2006; Rodd et al., 2003, 2006; Rodd-Henricks et al., 2000a,b, 2001; Serra et al., 2003; Vengeliene et al., 2005, 2006; Wolffgramm and Heyne, 1995), mice (Cowen et al., 2003a,b; Khisti et al., 2006; Melendez et al., 2006; Sanchis-Segura et al., 2006; Zghoul et al., 2007), monkeys (Kornet et al., 1990; Sinclair, 1971), and humans (Burish et al., 1981; Mello and Mendleson, 1972). The ADE can be seen at ethanol deprivation intervals as short as 12 hours (Sinclair et al., 1989) or as long as 75 days (Sinclair et al., 1973), and has been shown to increase in magnitude and duration following multiple cycles of ethanol deprivation in alcohol preferring (P) rats and high alcohol drinking (HAD) rats (Breese et al., 2004; McKinzie et al., 1998; Rodd et al., 2003; Rodd-Henricks et al., 2001, 2002a,b). Importantly, ADE ethanol drinking appears to be truly "uncontrolled" as rats will continue to drink increased amounts of ethanol that are adulterated with aversive tastes such as quinine (Spanagel et al., 1996).

Ingestive behavior (i.e., feeding and drinking) is complex and may be divided into at least 2 components. Appetitive behaviors are those used to locate and acquire stimuli (e.g., food and water) in the environment while consummatory behaviors are those used to directly consume the stimuli once they have been obtained (Samson and Hodge, 1995). Previous experiments evaluating the ADE in mice have primarily measured consummatory behavior, that is, the mice engaged in simple consumption of the ethanol solution from a sipper tube that extended into the cage after a period of imposed ethanol abstinence. Operant procedures allow for the analysis of consummatory behavior as well as appetitive or "seeking" responses (i.e., lever pressing is required to gain access to the ethanol solution). The distinction between appetitive and consummatory behavior has a useful clinical application. Some human alcoholics report a subjective "craving" component toward alcohol (Jellinek, 1955) which may ultimately drive intentional behaviors involved in obtaining access to alcohol (i.e., the appetitive component). Additionally, alcoholism is thought to entail loss of control over ethanol drinking (Marlatt and George, 1984) once consumption has been initiated (i.e., the consummatory component). Furthermore, drugs acting on dopamine or glutamatergic receptors have been found to uniquely influence consummatory or appetitive behaviors associated with ethanol ingestion (Czachowski et al., 2001a,b, 2002). Because different neuronal pathways appear to modulate appetitive versus consummatory behaviors during ethanol self-administration, and because ADE studies in mice have relied on bottle drinking procedures, 1 goal of the present project was to determine if a reliable ADE could be observed in C57BL/6J mice using operant self-administration procedures.

A second goal of the present report was to further characterize the neurochemical substrate involved in modulating the ADE. Previous studies utilizing pharmacologic approaches have implicated the dopamine D3 (Vengeliene et al., 2006), glutamate (Backstrom et al., 2004a; Holter and Spanagel, 1999; Rodd et al., 2006; Sanchis-Segura et al., 2006; Spanagel et al., 1996; Vengeliene et al., 2005), and opioid (Holter et al., 2000) receptors in signaling of ADE drinking. Another interesting target is corticotropin releasing factor (CRF), a 41 amino acid polypeptide with high concentrations in the hypothalamus, the brainstem, and the amygdala (Swanson et al., 1983). Both acute and chronic ethanol exposure activate central CRF pathways (Koob et al., 1993; Rasmussen et al., 2000; Rivier et al., 1984). Increased levels of CRF are observed in the amygdala during ethanol withdrawal (Merlo Pich et al., 1995) and the anxiogenic effect of ethanol withdrawal is reversed by CRF receptor antagonists (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2004; Rassnick et al., 1993). Of critical interest, administration of CRF receptor antagonists have been shown to attenuate excessive ethanol drinking in ethanol-dependent rodents without influencing ethanol intake by nondependent animals (Chu et al., 2007; Finn et al., 2007; Funk and Koob, 2007; Funk et al., 2006, 2007; Gehlert et al., 2007; Overstreet et al., 2007; Valdez et al., 2002). These observations suggest that central CRF receptor signaling modulates increased ethanol drinking in dependent animals and thus make CRF a possible candidate in the modulation of ADE drinking. To address this question, we studied the expression of ADE behavior in C57BL/6J mice following administration of the CRF-1 receptor antagonist, CP-154,526. Data from this report suggest that the ADE can be modeled in C57BL/6J mice using an operant self-administration paradigm, and that expression of the ADE is modulated by CRF-1 receptor signaling.

METHODS

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were 6 to 8 weeks old and weighed between 25 and 30 g at the start of all experiments and were single housed in polypropylene cages with corncob bedding and ad libitum access to food and water. Standard rodent chow (Teklad, Madison, WI) and water were available at all times except where noted. The vivarium rooms were maintained at an ambient temperature of 22°C with a 12-hour/12-hour light–dark cycle. All experimental procedures were approved by the University of North Carolina Animal Care and Use Committee (IACUC) and complied with the NIH Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

Drugs

CP-154,526 [butyl-(2,5-dimethyl-7-[2,4,6-trimethylphenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-ethylamine] was donated by Pfizer (Groton, CT), and was suspended in a vehicle of 0.5% carboxymethylcellulose (CMC). CP-154,526 displays high affinity for the CRF-1 receptor (K_i < 10 nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al., 1996; Schulz et al., 1996). Peripheral administration of CP-154,526 crosses the blood–brain barrier and reaches peak brain concentrations 20 minutes after administration with significant levels of the drug observed in the cortex, striatum, cerebellum, and hippocampus (Keller et al., 2002). Importantly, previous research found that systemic administration of a

10 mg/kg dose of CP-154,526 effectively reduced anxiety-like behavior in mice (Griebel et al., 1998). During operant training (see below), mice received daily intraperitoneal (i.p.) injections of 0.5% CMC (5 ml/kg) 30 minutes before operant sessions to habituate them to injection procedures (except in Experiment 4). Injection site was alternated between sides (left or right) daily to minimize tissue damage.

Operant Training

Self-administration experiments were conducted in 16 modular mouse operant chambers (Med Associates, Georgia, VT) with dimensions of 21.6 × 17.8 × 12.7 cm and a stainless steel grid floor. All chambers were housed in a sound-attenuating shell with a ventilation fan. Liquid receptacles were located in the center of the right and left chamber walls and a stainless steel response lever was to the right of each receptacle. Liquid solutions (primary lever produced sucrose or ethanol and a second lever produced water or was inactive) were infused using 10 ml plastic syringes which were mounted on a programmable pump (PHM-100, Med Associates, Georgia, VT). The pump delivered 0.01 ml of solution per activation. A yellow stimulus light and tone (80 dB) were activated when the primary lever (sucrose/ethanol reinforced) was depressed (except in Experiment 3 below). No stimulus light or tone occurred when the second lever (water reinforced or inactive) was pressed. A house light inside the operant chambers was on for the duration of the test. Data recorded during each 2-hour operant session included the number of sucrose/ethanol- and water-reinforced (or inactive) responses (bar presses), the number of sucrose/ethanol and water reinforcers (pump activation), and ethanol intake (g/kg body weight). The operant chambers were interfaced to an IBM computer and all data were automatically recorded using Med Associates software (MED-PC for Windows®, Version IV; Med Associates, Georgia, VT). All operant sessions were completed in the light phase of the light/dark cycle.

Mice were placed under a modified operant sucrose fading procedure (Samson, 1986; Schroeder et al., 2003). Briefly, mice were initially trained to respond to the levers. Responses to the primary lever resulted in the delivery of a 10% sucrose solution (w/v) and responses to the second lever caused delivery of distilled water (or was inactive). Mice were allowed to respond for 10% sucrose for 4 days in 16-hour sessions to strengthen lever pressing behavior. Sessions were then reduced to 2 hours per day for the remainder of the experiment. Following stable responding (i.e., no significant differences in responding over 3 consecutive days), increasing concentrations of ethanol were introduced to the 10% sucrose solution every 2 days [2, 4, 8, and 10% ethanol (v/v)]. Then, the sucrose concentration was reduced every 2 days (5, 2, and 0% sucrose) until mice were responding only for 10% ethanol. From the point at which ethanol was introduced into the sucrose solution onward, mice were given access to 2 bottles in their homecages (1 containing water and the other contain an ethanol solution). The ethanol concentration presented in the homecage matched the concentration of ethanol being tested in the operant chambers. Thus, animals had access to ethanol for 24-hours per day during the ethanol training phase to prevent ethanol deprivation. Once mice displayed stable responding for 10% ethanol (approximately 2 weeks of training), ADE sessions were initiated. Lever responding over the last 3 days of training were averaged for each mouse and served as their baseline (BL) response rate.

Experiment 1: Effect of Ethanol Deprivation-Induced Lever Responding With Water Reinforced Alternate Response Lever

Immediately following BL sessions, mice ($n = 32$) were not run in the operant chambers and homecage ethanol was removed for a 4-day ethanol deprivation period. Following the deprivation period, mice were tested in daily 2-hour operant sessions over 3 consecutive days and given access to 10% ethanol in their homecages immediately after the first postdeprivation operant session. Ethanol deprivation and access were repeated, and mice were then used in Experiment 2.

Experiment 2: Effect of CP-154,526 Administration on Ethanol Deprivation-Induced Lever Responding With Water Reinforced Alternate Response Lever

Following 4 days of BL responding, mice were deprived of ethanol for 4 days (no operant sessions and no homecage ethanol access). Mice were distributed to 3 groups matched for BL lever responding and given i.p. injection of 0, 10, or 20 mg/kg doses of CP-154,526 30 minutes before the test session which immediately followed the 4-day deprivation period. The effects of CP-154,526 on deprivation-induced ethanol reinforced lever pressing was then assessed over the 2-hour operant test session. After a second 4-day deprivation period, mice previously injected with the 0 and 10 mg/kg doses of CP-154,526 (with the exception of 2 mice that became sick) were injected with the other dose 30-minutes before a second 2-hour ADE test session. Mice previously injected with the 20 mg/kg dose were not tested a second time due to the apparent aversive effects induced by this high dose of CP-154,526. At the end of the study, the sample size for the 0, 10, and 20 mg/kg groups were $n = 25$, 27, and 10, respectively.

Experiment 3: Ethanol Deprivation-Induced Lever Responding With Inactive Alternate Response Lever

To further characterize the ADE using operant procedures, male C57BL/6J mice ($n = 14$) were trained as described above with 2 exceptions: the second lever was inactive such that responses were not reinforced, and no tone or light were presented when the primary lever was activated. Following BL responding, mice were deprived of ethanol for 4-days (no operant procedures and no home cage ethanol access). Mice were then tested in 2-hour operant sessions over 3 consecutive days along with homecage access to ethanol.

Experiment 4: Effect of CP-154,526 Administration on Sucrose Deprivation Testing With Water Reinforced Alternate Response Lever

Male C57BL/6J mice ($n = 15$) were tested to determine if a 4-day deprivation and pretreatment with CP-154,526 would alter lever pressing reinforced with 1% (w/v mixed in tap water) sucrose. The 1% sucrose solution was chosen because it promoted similar levels of lever pressing obtained with 10% ethanol reinforcement during 2-hour test sessions. Responding to the primary lever was reinforced with 1% sucrose and the second lever was reinforced with water. During training, mice were also given 1% sucrose in addition to water in their home cages. After a stable BL for sucrose responding was established, mice were distributed into 2 groups matched for BL sucrose responding. Following a 4-day sucrose deprivation period (no operant procedures and no home cage sucrose access), mice were injected with 0 ($n = 7$) or a 10 mg/kg dose of CP-154,526 ($n = 8$) 30 minutes before a 2-hour operant test session.

Experiment 5: Ethanol Deprivation-Induced Lever Responding With Water Reinforced Alternate Response Lever in Mice not Given i.p. Injections

To determine if the stress associated with daily i.p. injections in Experiment 1–4 may have altered the overall level of lever pressing, mice were tested in the operant self-administration paradigm in the absence of i.p. injections. Briefly, male C57BL/6J mice ($n = 32$) were trained to press levers for ethanol or water reinforcement as described above except i.p. injections were never administered. Once stable responding occurred for the 10% ethanol solution, mice were

not run in the operant chambers and homecage ethanol was removed for a 4-day break. Mice were then tested in 2-hour operant sessions over 3 consecutive days with access to 10% ethanol in their homecages as described above.

Data Analysis

All data in this report are presented as means ± SEM. We used analyses of variance (ANOVA) to analyze data from each experiment. When significant effects were obtained, we performed planned comparisons with paired or independent *t*-tests (Winer et al., 1991). Significance was accepted at $p < 0.05$ (2-tailed).

RESULTS

Experiment 1: Effect of Ethanol Deprivation-Induced Lever Responding With Water Reinforced Alternate Response Lever

Figure 1*A* depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice at BL (last 3 sessions before the first ethanol deprivation cycle) and during the 3 sessions of postdeprivation responding following the first and a repeated deprivation session. A 2-way mixed-factor ANOVA run on 10% ethanol lever response data indicated a significant main effect of session [$F(3,186) = 22.42$, $p < 0.01$] and a significant session × deprivation cycle interaction [$F(3,186) = 3.80$, $p = 0.01$]. Following the first ethanol deprivation cycle, planned comparisons revealed that mice performed significantly more responses for 10% ethanol on the first postdeprivation session relative to BL ethanol lever responding ($t = 3.49$, $p < 0.01$). Following the repeated ethanol deprivation cycle, the rate of ethanol lever pressing on the first, second, and third postdeprivation sessions were significantly higher when compared to the BL ethanol lever responding ($t = 6.68$, $p < 0.01$; $t = 4.24$, $p < 0.01$; $t = 3.17$, $p = 0.03$, respectively). Mean lever responses for water at BL and during the 2-hour postdeprivation sessions are shown in Fig. 1*B*. A 2-way mixed-factor ANOVA run on water data indicated a significant main effect of session [$F(3,186) = 16.95$, $p < 0.01$] and a significant session × deprivation cycle interaction [$F(3,186) = 5.24$, $p < 0.01$]. Following the first ethanol deprivation cycle, planned comparisons revealed that water lever pressing on the first session of operant testing was significantly higher than the BL water response rate ($t = 4.67$, $p < 0.01$). Following the repeated ethanol deprivation cycle, lever pressing for water on the first, second, and third postdeprivation sessions were significantly higher when compared to the BL water lever responding ($t = 4.96$, $p < 0.01$; $t = 2.98$, $p = 0.01$; $t = 2.82$, $p = 0.01$, respectively).

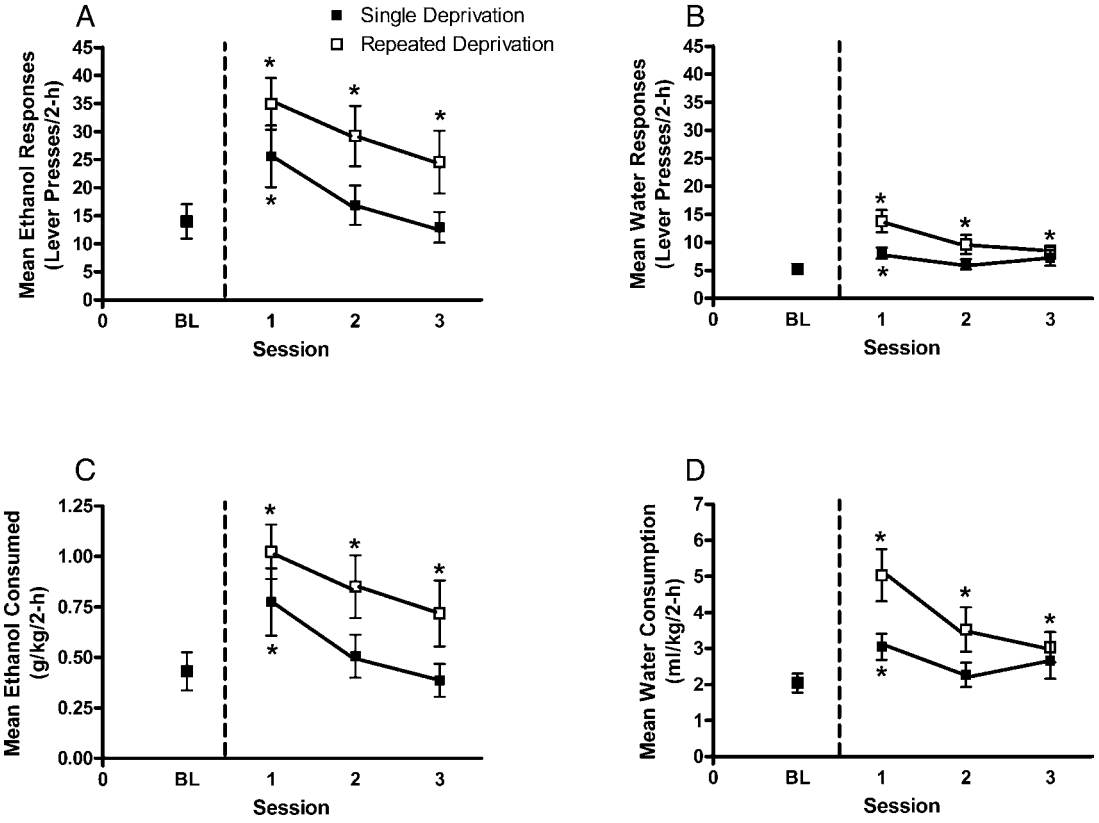

Fig. 1. Lever responses for 10% (v/v) ethanol (A) and water (B) during the 2-hour test sessions following the first 4-day ethanol deprivation cycle (deprivation 1) and after a repeated ethanol deprivation cycle (repeated deprivation). Consumption of 10% (v/v) ethanol (g/kg) (C) and water (ml/kg) (D) during the 2-hour test sessions following the first and repeated ethanol deprivation cycles. Baseline (BL) refers to the average of the last 3 sessions before alcohol deprivation effect procedures were introduced. All values are means ± SEM. *$p < 0.05$ relative to BL measures.

Figures 1*C*,*D* present the amount of ethanol (g/kg) and water (ml/kg) consumed by mice, respectively. A 2-way mixed-factor ANOVA run on ethanol consumption data revealed a significant main effect of session [$F(3,186) = 21.11$, $p < 0.01$] and a significant session × deprivation cycle interaction [$F(3,186) = 3.38$, $p = 0.02$]. Following the first ethanol deprivation cycle, mice consumed significantly more ethanol relative to BL following the first postdeprivation session ($t = 3.39$, $p < 0.01$). Following the repeated ethanol deprivation cycle, mice consumed more ethanol relative to BL during each of the 3 postdeprivation sessions ($t = 6.34$, $p < 0.01$; $t = 4.07$, $p < 0.01$; $t = 2.90$, $p < 0.01$). Similarly, a 2-way mixed-factor ANOVA run on water consumption data revealed a significant main effect of session [$F(3,186) = 16.81$, $p < 0.01$] and a significant session × deprivation cycle interaction [$F(3,186) = 4.81$, $p < 0.01$]. Following the first deprivation cycle, mice showed elevated water consumption relative to BL during the first postdeprivation session ($t = 4.66$, $p < 0.01$), and following the repeated deprivation cycle water consumption was significantly elevated above BL levels during each of the 3 sessions ($t = 4.48$, $p < 0.01$; $t = 2.76$, $p = 0.01$; $t = 2.55$, $p = 0.02$).

Experiment 2: Effect of CP-154,526 Administration on Ethanol Deprivation-Induced Lever Responding With Water Reinforced Alternate Response Lever

To determine if CRF-1 receptor signaling modulates deprivation-induced increases of ethanol-reinforced lever pressing, mice were pretreated with the CRF-1 receptor antagonist CP-154,526 30 minutes before testing. Figure 2*A* depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice during BL and on the postdeprivation session in which mice were administered CP-154,526 (0, 10, 20 mg/kg) 30 minutes before operant testing. A 1-way ANOVA comparing each of the 4 conditions was significant [$F(3,90) = 6.044$, $p = 0.001$]. Consistent with the ADE, mice showed significantly greater postdeprivation lever responding following administration of the vehicle when compared to their BL ethanol lever response rate ($t = 2.07$, $p = 0.044$). Importantly, there was no significant difference between BL ethanol responding and postdeprivation ethanol responding when mice were administered the 10 mg/kg dose of CP-154,526. However, the 20 mg/kg dose of CP-154,526 significantly reduced 10% ethanol lever responding relative to BL ($t = 2.458$, $p = 0.018$). Figure 2*B* depicts the mean lever

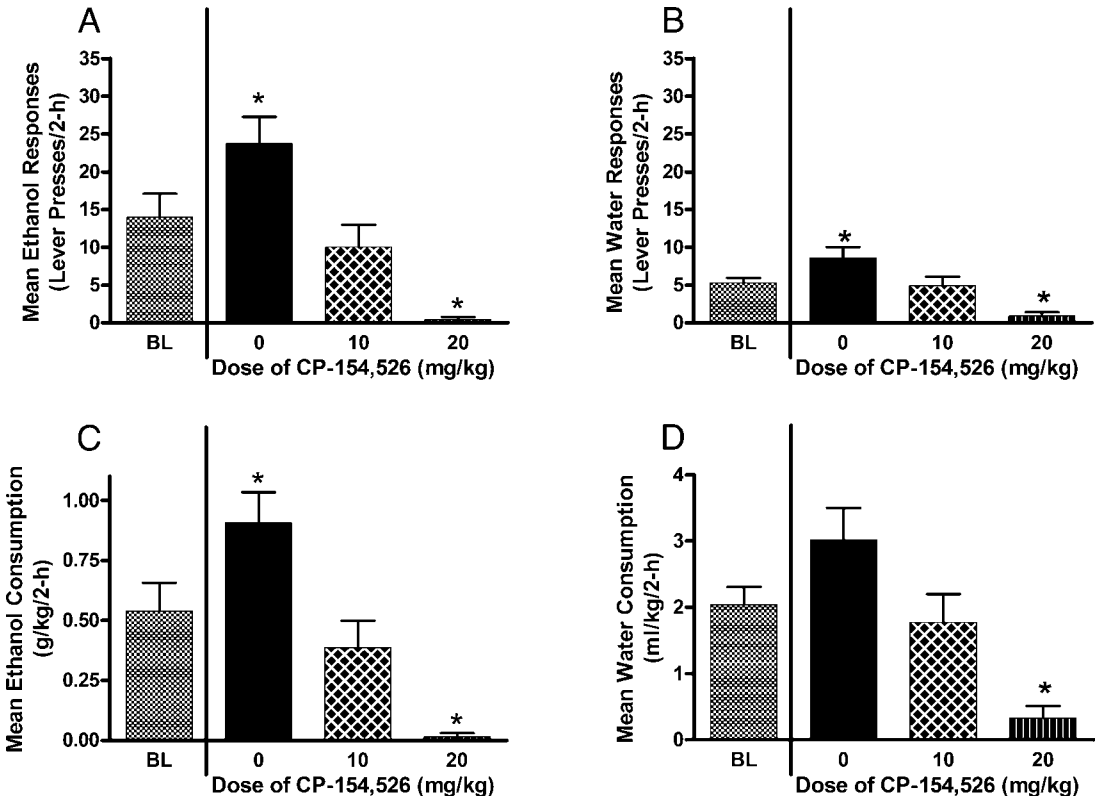

Fig. 2. Lever responses for 10% (v/v) ethanol (A) and water (B), and consumption of ethanol (C) and water (D) during the 2-hour test immediately following 4-days of ethanol deprivation. Mice were given an intraperitoneal injection of the corticotropin releasing factor-1 receptor antagonist CP-154,526 (0, 10, 20 mg/kg) 30 minutes before testing. Baseline (BL) refers to the average of the last 3 sessions before alcohol deprivation effect procedures were introduced. All values are means ± SEM. *$p < 0.05$ relative to BL measures.

responses for water during BL and on the postdeprivation session following administration of CP-154,526 (0, 10, 20 mg/kg). A 1-way ANOVA run on the data was significant [$F(3,90) = 4.94$, $p = 0.003$]. The vehicle treated group had a significantly greater number of water lever responses when compared to the BL water lever response rate ($t = 2.18$, $p = 0.034$). Relative to BL, there was no significant difference in water responding following treatment with the 10 mg/kg dose of CP-154,526, and the 20 mg/kg dose of CP-154,526 significantly reduced water lever responding relative to BL ($t = 3.424$, $p = 0.001$).

Figure 2*C* depicts the mean consumption of 10% ethanol (g/kg/2-h session) by C57BL/6J. A 1-way ANOVA comparing each of the 4 conditions was significant [$F(3,90) = 4.903$, $p < 0.003$]. Planned comparisons with 2-tailed *t*-tests revealed that groups treated with vehicle or the 10 mg/kg dose of CP-154,526 did not significantly differ in postdeprivation ethanol consumption relative to BL intake. As we predicted a significant increase of ethanol consumption following ethanol deprivation, we performed a directional 1-tailed *t*-test and found that the vehicle treated group showed a significant deprivation-induced increase of ethanol consumption relative to BL levels ($t = 1.719$, $p = 0.0456$). The 20 mg/kg dose of CP-154,526 significantly reduced 10% ethanol intake relative to BL ($t = 2.458$, $p = 0.018$). Figure 2*D* depicts water consumption (ml/kg/2-h session) by the C57BL6J mice. A 1-way ANOVA run on the data was significant [$F(3,90) = 4.903$ $p = 0.003$]. The only significant planned comparison showed that the 20 mg/kg dose of CP-154,526 significantly reduced water consumption relative to BL ($t = 3.503$, $p = 0.001$).

Experiment 3: Ethanol Deprivation-Induced Lever Responding With Inactive Alternate Response Lever

Unexpectedly, there was a deprivation-induced increase of water-reinforced lever responding in Experiments 1 and 2. To determine if increased responding on the water-reinforced lever may have resulted from a general nonspecific increase in activity following the deprivation sessions, Experiment 3 examined the ADE using operant procedures but with the secondary lever inactive (nonreinforced). We reasoned that nonspecific increases of activity resulting from a deprivation period should also promote increased responding to a nonreinforced lever. Figure 3*A* depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice at BL (last 3 sessions before the first ethanol deprivation cycle) and the 3 sessions of postdeprivation responding following the 4-day deprivation. A repeated measures ANOVA comparing BL responding and the 3 days of postdeprivation responding was significant [$F(3,39) = 3.671$, $p = 0.020$]. Planned comparisons revealed that mice performed significantly more responses for 10% ethanol on the first and second (but not third) postdeprivation session relative to BL ethanol lever responding ($t = 2.434$, $p = 0.030$; $t = -2.902$, $p = 0.012$). Figure 3*B* shows mean ethanol consumption (g/kg/2-h) during this study. A repeated measures ANOVA

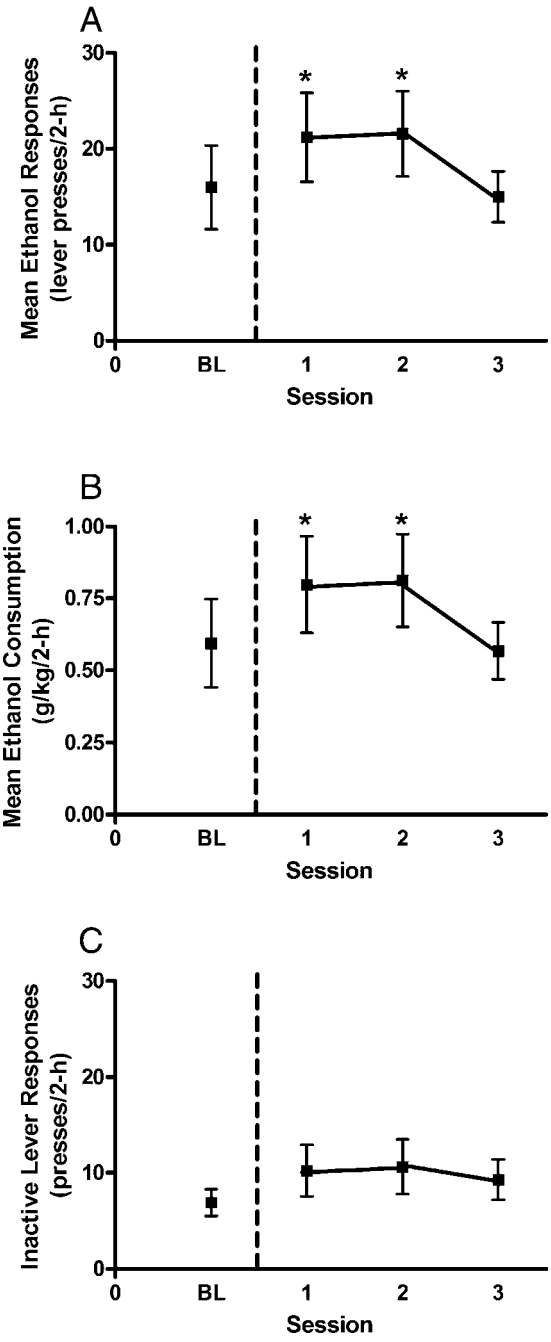

Fig. 3. Lever responses for 10% (v/v) ethanol (A) ethanol consumption (B), and responses on the inactive lever (C) during the 2-hour test sessions following the 4-day ethanol deprivation cycle. Baseline (BL) refers to the average of the last 3 sessions before alcohol deprivation effect procedures were introduced. All values are means ± SEM. $p < 0.05$ relative to BL measures.

comparing BL ethanol intake and the 3 days of postdeprivation ethanol consumption was significant [$F(3,39) = 3.920$, $p = 0.015$], and planned comparisons revealed that mice consumed significantly more 10% ethanol on the first and second (but not third) postdeprivation session relative to BL ethanol intake ($t = 2.481$, $p = 0.028$; $t = 3.009$, $p = 0.010$). Figure 3*C* shows mean responses to the inactive lever during

each 2-hour session. A repeated measures ANOVA performed on these data did not achieve statistical significance [$F(3,39) = 1.145$, $p = 0.343$].

Experiment 4: Effect of CP-154,526 Administration on Sucrose Deprivation Testing with Water Reinforced Alternate Response Lever

To determine if the ability of CP-154,526 to attenuate deprivation-induced lever responding was specific to ethanol reinforcement, we determined if the 10 mg/kg dose of this CRF-1 receptor antagonist would attenuate lever responding reinforced with 1% sucrose solution following a 4-day deprivation period. Figure 4*A* shows mean sucrose reinforced lever pressing (over 2 hours) during BL and on the session immediately after the 4-day sucrose deprivation period, while Fig. 4*B* shows mean water reinforced responding on the second lever during the same sessions. Two-way mixed factor ANOVAs performed on ethanol- and water-reinforced lever responding data failed to show significant main effects of session (BL vs. postdeprivation) or CP-154,526 dose (0 or 10 mg/kg) or significant interaction effects. Figures 4*C*,*D* show mean sucrose and water consumption during the test, respectively. Similar to lever responding data, 2-way mixed factor ANOVAs performed on consumption data failed to achieve statistical significance.

Experiment 5: Ethanol Deprivation-Induced Lever Responding with Water Reinforced Alternate Response Lever in Mice not given i.p. Injections

While we observed a deprivation-induced increase of ethanol self-administration in Experiment 1, the amount of ethanol consumed after repeated deprivations was only about 1.0 g/kg over the 2-hour test. Since mice were periodically given i.p. injections in the experiments above, the present experiment determined if the level of ethanol-reinforced responding and ethanol intake would be higher in mice that did not experience injections during the study. Figure 5*A* depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice at BL (last 3 sessions before the first ethanol deprivation cycle) and the 3 sessions of postdeprivation responding following the 4-day deprivation period. A 1-way repeated measures ANOVA performed on 10% ethanol lever responding data was significant [$F(3,93) = 8.786$, $p < 0.001$]. Planned comparisons revealed that mice performed significantly more responses for 10% ethanol on the first postdeprivation session relative to BL ethanol lever responding ($t = 4.449$, $p < 0.001$). Mean lever responses for water at BL and during the 2-hour postdeprivation sessions are shown in Fig. 5*B*. A 1-way repeated measures ANOVA performed on water lever responding data was significant [$F(3,93) = 6.460$, $p = 0.001$] Water lever pressing

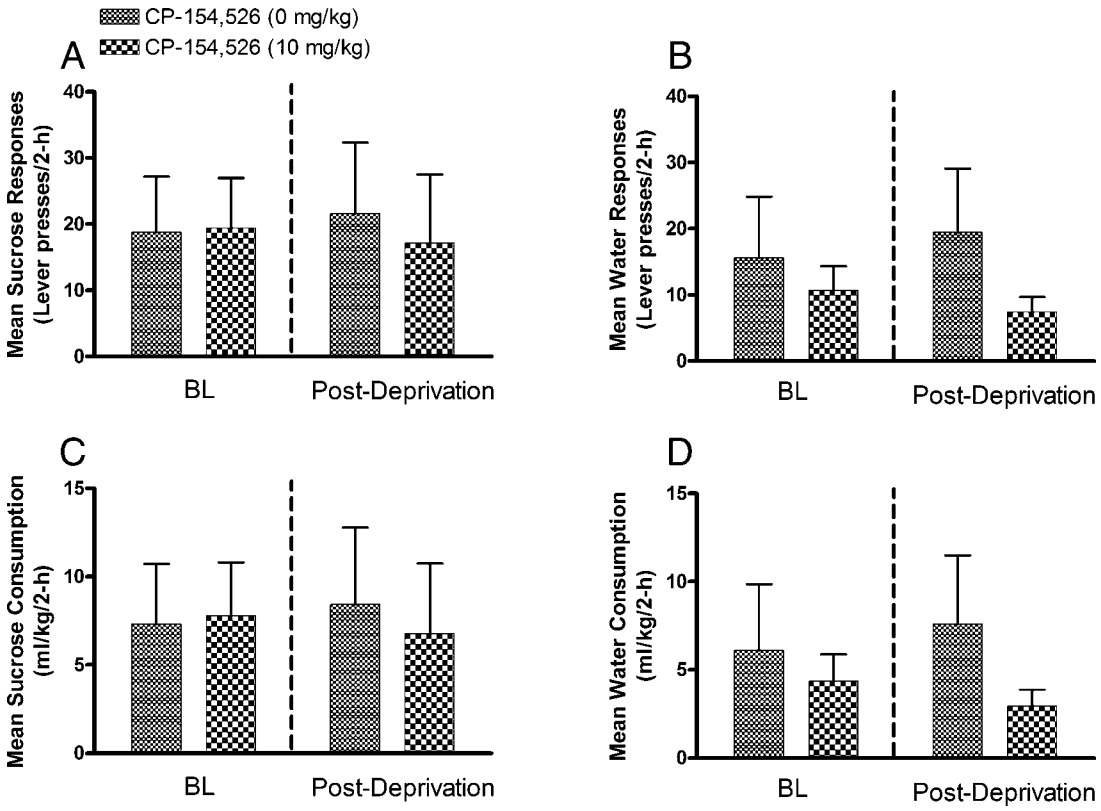

Fig. 4. Lever responses for 1% (w/v) sucrose (A) and water (B), and consumption of sucrose (C) and water (D) during the 2-hour test session following the 4-day sucrose deprivation cycle. Baseline refers to the average of the last 3 sessions before the 4-day deprivation procedure. On the test day (postdeprivation) mice were given intraperitoneal injection of the corticotropin releasing factor-1 receptor antagonist CP-154,526 (0 or 10 mg/kg) 30 minutes before testing. All values are means ± SEM.

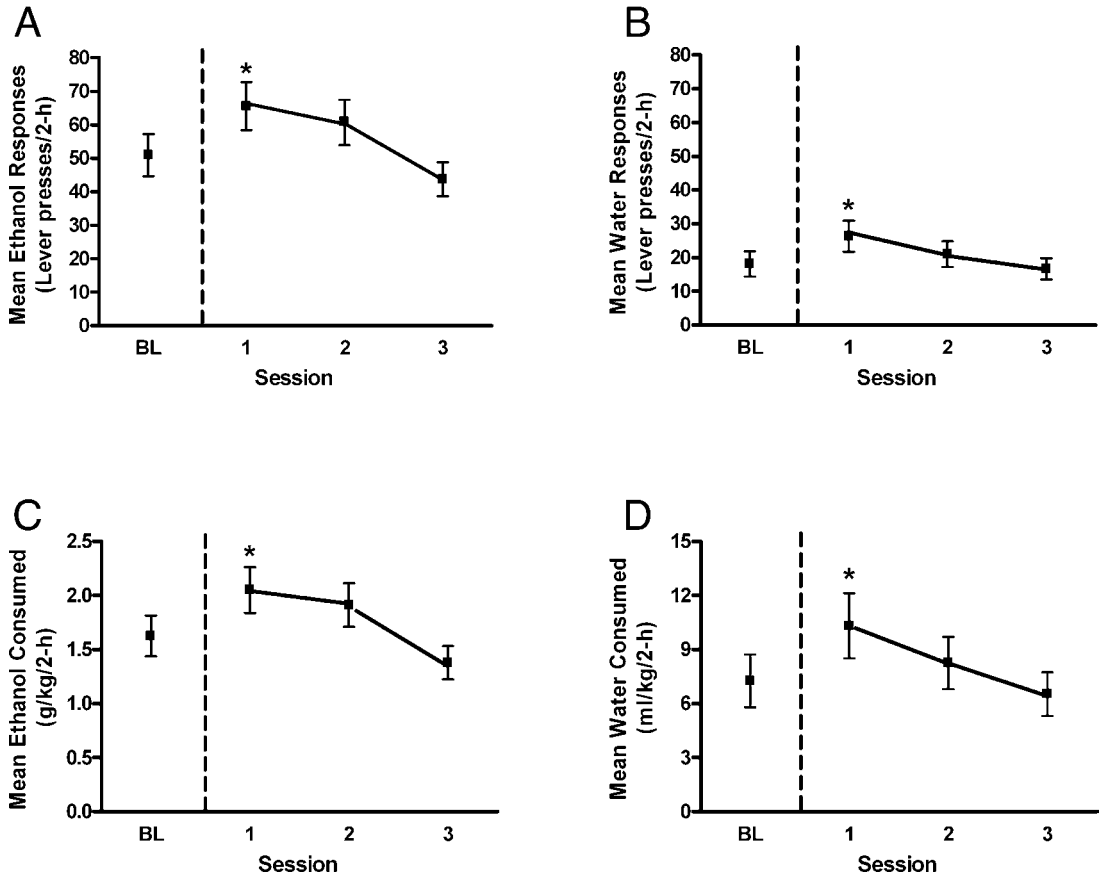

Fig. 5. Lever responses for 10% (v/v) ethanol (A) and water (B), and consumption of ethanol (C) and water (D) during the 2-hour test sessions following the 4-day ethanol deprivation cycle. Baseline (BL) refers to the average of the last 3 sessions before alcohol deprivation effect procedures were introduced. All values are means ± SEM. $p < 0.05$ relative to baseline measures.

on the first postdeprivation session of operant testing was significantly higher than the BL water response rate ($t = 3.595$, $p = 0.001$).

Figures 5*C*,*D* present the amount of ethanol (g/kg) and water (ml/kg) consumed by mice, respectively. A repeated measures ANOVA performed on ethanol consumption data was significant [$F(3,93) = 8.736$, $p < 0.001$], and planned comparisons showed that mice consumed significantly more ethanol relative to BL following the first postdeprivation session ($t = 4.065$ $p < 0.001$). Here, mice consumed approximately 2.0 g/kg of ethanol during the first 2-hour postdeprivation test session. Similarly, a repeated measures 1-way ANOVA performed on water consumption data revealed a significant effect [$F(3,93) = 5.975$, $p = 0.001$] and a planned comparison showed elevated water consumption relative to BL during the first postdeprivation session ($t = 3.344$, $p = 0.002$).

DISCUSSION

The present investigation shows that the ADE can be achieved with C57BL/6J mice using operant self-administration procedures. These observations add to the literature by showing that the ADE is associated with increased appetitive ethanol-seeking behavior (i.e., lever pressing to gain access to ethanol reinforcement) as well as increased consummatory behavior (ethanol consumption) when ethanol is returned after a period of forced abstinence in C57BL/6J mice. These findings are consistent with the rat literature in which the ADE has been observed using both bottle drinking and operant self-administration procedures (e.g., Overstreet et al., 2007; Rodd-Henricks et al., 2001, 2000a; Rodd et al., 2003; Toalston et al., 2008). Secondly, we show that pretreatment with a CRF-1 receptor antagonist protects against deprivation-induced increases of ethanol self-administration, an outcome evidenced by the observation the mice pretreated with the 10 mg/kg dose of CP-154,526 showed levels of ethanol-reinforced lever pressing after 4 days of ethanol deprivation that were similar to BL levels (Experiment 2). These results suggest that CRF-1 receptor signaling modulates the ADE in C57BL/6J mice.

One surprising observation in the present set of experiments was that the deprivation procedure caused an increase of water-reinforced lever pressing that paralleled ethanol-reinforced responding. This observation may suggest that the deprivation procedure employed here promoted a general increase in activity when mice were returned to the operant chambers, or that the deprivation procedure nonspecifically

enhanced the reinforcing value of both ethanol and water. Both of these possibilities are unlikely for 2 reasons. First, when the second operant lever was inactive (nonreinforced) in Experiment 3, mice displayed deprivation-induced increases of ethanol-reinforced lever responding but no significant increase of responding to the inactive key. If the deprivation procedure caused a general increase of activity when mice were returned to the operant chambers, inactive lever pressing would be expected to significantly increase, which did not happen (although there was a modest nonsignificant elevation on postdeprivation days 1 and 2). Second, the deprivation procedure did not lead to increased sucrose-reinforced lever pressing, a finding indicating that deprivation does not promote a nonspecific enhancement of reinforcer value. The sucrose study also provides additional evidence against deprivation-induced increases of general activity. One likely explanation for deprivation-induced increases of water-reinforced responding is that since ethanol is a diuretic agent, the increased motivation to gain access to water in mice with elevated ethanol self-administration may be due to thirst resulting from dehydration. Consistent with this argument, deprivation-induced increase of water-reinforced responding only occurred when ethanol served as the reinforcer for the primary lever (Figs. 1, 2, and 5), but not when sucrose was used as the reinforcer on the primary lever (Fig. 4).

In addition to demonstrating that the present ADE procedures do not promote deprivation-induced increases of sucrose-reinforced behavior, the sucrose control study (Experiment 4) also demonstrates that the effects of the 10 mg/kg dose of CP-154,526 were specific to responding for ethanol. Thus, after a 4-day deprivation, mice pretreated with the 10 mg/kg dose of CP-154,526 showed levels of ethanol-reinforced responding that were similar to predeprivation levels, while vehicle treated mice demonstrated the characteristic ADE. On the other hand, the 10 mg/kg dose did not significantly alter sucrose-reinforced responding after a 4-day deprivation period. Furthermore, we have previously shown that the 10 mg/kg dose of CP-154,526 does not alter open-field locomotor activity in C57BL/6J mice over a 4-hour test (Sparta et al., 2008). These observations provide novel evidence suggesting that CRF-1 receptor signaling selectively modulates deprivation-induced increases of ethanol-seeking behavior rather than affecting ongoing behavior in general. However, since mice had experienced the ADE prior to the test with the CRF-1 receptor antagonist (in Experiment 1), it is unclear if CRF-1 receptor blockade would attenuate the ADE after an initial ethanol deprivation. It should be noted that the 20 mg/kg dose of CP-154,526 likely produced nonspecific behavioral side-effects as this dose reduced ethanol-reinforced responding below levels observed in vehicle treated mice and below predeprivation BL levels.

With the present procedures, activation of the primary lever caused a brief activation of light and tone conditioned stimuli (CSs) that occurred concurrently with reinforcer presentation. One potential concern is that these CSs may have acquired conditioned secondary reinforcer value, and thus deprivation-induced increases of lever pressing may have been driven by CS reinforcement rather than increased motivation to gain access to ethanol. There are 2 observations that argue against this possibility. First, no light/tone CSs were used in Experiment 3, yet deprivation-induced increases of ethanol-reinforced lever pressing were observed. On the other hand, the CSs were used in the sucrose control study (Experiment 4) where there were no observed increases of sucrose-reinforced lever pressing following deprivation. Thus, a role for the CSs in modulating deprivation-induced increases of ethanol-reinforced lever pressing seems unlikely.

In Experiment 1, mice consumed a little more than 1 g/kg/2-h during the first day of postdeprivation testing after repeated deprivation. To determine if the regular i.p. injections given to mice may have led to an overall reduction of ethanol-seeking behavior and consumption, mice were tested using the ADE procedures but without any i.p. injections in Experiment 5. Consistent with an inhibitory effect of injections on ethanol-reinforced responding, mice in Experiment 5 consumed approximately 2 g/kg/2-h of ethanol during the first test after the 4-day deprivation period. Based on previous research in which C57BL/6J mice consumed approximately 2 g/kg of ethanol over a 2-hour test (Rhodes et al., 2005), mice in Experiment 5 would have achieved blood ethanol levels of approximately 55 mg/ml following the first day of postdeprivation testing, although caution is necessary with respect to this blood ethanol estimate given the procedural differences between the Rhodes et al. work and the present study. Nonetheless, based on the previous bottle drinking studies (Rhodes et al., 2005), we speculate that longer test sessions (e.g., 4 hours) and testing within the animal's dark cycle would further increase deprivation-induced increases of ethanol-reinforced responding.

While procedures involving bottle drinking allow for the analysis of factors that modulate consummatory behavior, it has been suggested that operant procedures allow for the analysis of appetitive or "ethanol-seeking" behavior (measured by lever pressing) as well as consummatory behavior (Samson and Hodge, 1995). Previous work with mice using bottle drinking procedures demonstrate that deprivation-induced increases of ethanol intake results from increased levels of consummatory behavior (Cowen et al., 2003a,b; Khisti et al., 2006; Melendez et al., 2006; Sanchis-Segura et al., 2006; Zghoul et al., 2007). Using operant self-administration procedures, here we show that the ADE also involves increased appetitive "ethanol-seeking" behavior. Because different neuronal pathways appear to modulate appetitive versus consummatory behaviors during ethanol self-administration (Czachowski et al., 2001a,b, 2002; Ford et al., 2007), the combined use of bottle drinking and operant self-administration procedures will allow for a more complete characterization of the neurobiological mechanisms underlying the ADE. Here we show that CRF-1 receptor signaling modulates appetitive components of the ADE. It will be interesting to determine if

CRF-1 receptor antagonists also modulate deprivation-induced increases of ethanol intake using bottle drinking procedures.

The present findings are consistent with previous data that have revealed a role for CRF receptor signaling in neurobiological responses to ethanol. First, increased levels of CRF are observed in the amygdala during ethanol withdrawal (Merlo Pich et al., 1995) while the anxiogenic effect of ethanol withdrawal is reversed by CRF receptor antagonists (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2004; Rassnick et al., 1993). Second, antagonism of CRF receptors attenuates increased ethanol drinking in rodents made dependent to ethanol by exposure to ethanol diet or ethanol vapor, but has no effect on moderate levels of ethanol consumption in nondependent rodents (Chu et al., 2007; Finn et al., 2007; Funk and Koob, 2007; Funk et al., 2006, 2007; Gehlert et al., 2007; Valdez et al., 2002). Recently, we found that pretreatment with CP-154,526 prevents binge-like ethanol drinking in C57BL/6J mice (Sparta et al., 2008). Third, stress-induced reinstatement of operant ethanol self-administration (an animal model of ethanol relapse) is blocked by administration of a CRF receptor antagonist and increased by central infusion of CRF (Le et al., 2000; Liu and Weiss, 2002; Stewart, 2004). Taken together with the present results, a picture emerges such that CRF receptor signaling appears to be part of a dynamic mechanism that is involved with the development of ethanol dependence stemming from repeated ethanol exposure and withdrawal, a mechanism illustrated by the recently proposed allostasis and "kindling"/stress models of drug dependence (Breese et al., 2005; Koob, 2003; Koob and Le Moal, 2001).

In conclusion, we show here that the ADE in male C57BL/6J mice is observed using operant self-administration procedures. Importantly, we provide novel evidence that expression of the ADE in C57BL/6J mice may be modulated by the CRF-1 receptor. It will be important to determine if the CRF-1 receptor modulates the ADE using bottle drinking procedures, or if CRF-1 receptor signaling selectively modulates appetitive ethanol-seeking behaviors associated with the ADE as revealed in the present study. The present work adds to a growing body of literature implicating the CRF system in modulating neurobiological responses to ethanol, observations that together suggesting a possible therapeutic role for CRF-1 receptor antagonists in the treatment of alcoholism and the prevention of relapse.

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Blockade of the Corticotropin Releasing Factor Type 1 Receptor Attenuates Elevated Ethanol Drinking Associated With Drinking in the Dark Procedures

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Background: Drinking in the dark (DID) procedures have recently been developed to induce high levels of ethanol drinking in C57BL/6J mice, which result in blood ethanol concentrations (BECs) reaching levels that have measurable affects on physiology and/or behavior. The present experiments determined whether the increased ethanol drinking caused by DID procedures can be attenuated by pretreatment with CP-154,526; a corticotropin releasing factor type-1 (CRF_1) receptor antagonist.

Methods: In Experiment 1, male C57BL/6J mice received ethanol (20% v/v) in place of water for 4 hours, beginning with 3 hours into the dark cycle. On the fourth day, mice were given an intraperitoneal injection of one of the 4 doses of CP-154,526 (0, 1, 3, 10 mg/kg) 30 minutes before receiving their ethanol bottle. In Experiment 2, C57BL/6J mice had 2 hours of access to the 20% ethanol solution, beginning with 3 hours into the dark cycle on days 1 to 3, and 4 hours of access to the ethanol bottle on day 4 of DID procedures. Mice were given an intraperitoneal injection of one of the 4 doses of CP-154,526 (0, 1, 3, 10 mg/kg) 30 minutes before receiving their ethanol bottle on day 4. Tail blood samples were collected immediately after the 4-hour ethanol access period on the fourth day of each experiment. Additional control experiments assessed the effects of CP-154,526 on 4-hour consumption of a 10% (w/v) sucrose solution and open-field locomotor activity.

Results: In Experiment 1, the vehicle-treated group consumed approximately 4.0 g/kg/4 h of ethanol and achieved BECs of approximately 30 mg%. Furthermore, pretreatment with the CRF_1 receptor antagonist did not alter ethanol consumption. On the other hand, procedures used in Experiment 2 resulted in vehicle-treated mice consuming approximately 6.0 g/kg/4 h of ethanol with BECs of about 80 mg%. Additionally, the 10 mg/kg dose of CP-154,526 significantly reduced ethanol consumption and BECs to approximately 3.0 g/kg/4 h and 27 mg%, respectively, relative to vehicle-treated mice. Importantly, the 10 mg/kg dose of the CRF_1R antagonist did not significantly alter 4-hour sucrose consumption or locomotor activity.

Conclusions: These data indicate that CRF_1R signaling modulates high, but not moderate, levels of ethanol drinking associated with DID procedures.

Key Words: C57BL/6J Mice, Drinking in the Dark, Corticotropin Releasing Factor, CRF_1 Receptor, CP-154,526.

RODENT MODELS OF alcoholism, including inbred and selectively bred strains have been useful tools for identifying the genetic and neurobiological factors that underlie this disease. However, in many cases, rodents do not consume enough alcohol to reach the point of behavioral and/or pharmacological intoxication (Spanagel, 2000). Recently, "drinking in the dark" (DID) procedures have been developed to induce excessive ethanol drinking in C57BL/6J mice, which result in blood ethanol concentrations (BECs) reaching levels that have measurable effects on physiology and/or behavior (Rhodes et al., 2005, 2007). With these procedures, C57BL/6J mice are given access to a 20% ethanol solution for 2 to 4 hours, starting with 3 hours into their dark cycle. C57BL/6J can achieve BECs of > 100 mg% and exhibit signs of behavioral intoxication as measured by motor deficits on the rotarod and balance beam (Rhodes et al., 2005, 2007). It has been argued that the DID model has predictive validity for testing potential pharmacological targets aimed at treating alcohol abuse disorders as naltrexone, an opioid receptor antagonist which is currently used to treat alcoholism, dose dependently attenuates high levels of ethanol drinking induced by DID procedures (Kamdar et al., 2007).

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Corticotropin releasing factor (CRF) is a 41 amino acid neuromodulator that is widely expressed throughout the central nervous system (Bloom et al., 1982; Merchenthaler et al., 1982). CRF has been shown to modulate diverse biological functions including food intake, stress and anxiety-like behaviors, and neurobiological responses to ethanol [for reviews, see (Heilig and Koob, 2007; Valdez, 2006; Zorrilla and Koob, 2004; Zorrilla et al., 2003)]. Increases in CRF immunoreactivity (Olive et al., 2002; Zorrilla et al., 2001) and levels of extracellular CRF (Funk et al., 2006) are seen in the amygdala following ethanol withdrawal. Exposure to ethanol causes robust activation of the hypothalamic-pituitary-adrenal (HPA)-axis (Rivier, 1996; Rivier et al., 1990), which is initiated by ethanol-induced increases of CRF activity within the hypothalamus (Li et al., 2005; Rivier and Lee, 1996). Recent pharmacological and genetic evidence support the hypothesis that CRF exerts its effects on ethanol consumption through activation of the CRF_1 receptor (CRF_1R). Blockade of the CRF_1R attenuates ethanol intake in dependent, but not nondependent rodents (Funk et al., 2007; Gehlert et al., 2007). Consistently, CRF_1R deficient mice failed to show increased ethanol consumption following the acquisition of ethanol dependence and a period of abstinence that was observed in wild-type mice (Chu et al., 2007). Interestingly, a genetic polymorphism at the Crhr1 locus, which encodes the CRF_1R was found to be significantly linked to alcoholism (Treutlein et al., 2006).

Because CRF receptor signaling has been implicated in a wide range of neurobiological responses to ethanol, the goal of the present set of experiments was to determine whether the increased consumption of ethanol associated with DID procedures can be modulated by pretreatment with CP-154,526, a CRF_1R antagonist. Specifically, because ethanol triggers HPA-axis signaling which is initiated by ethanol-induced increases of CRF activity within the hypothalamus (Li et al., 2005; Rivier and Lee, 1996), and because levels of corticosterone, a HPA-axis-associated hormone, have been shown to positively correlated with ethanol intake (Fahlke et al., 1994, 1995, 1996), we predicted that CRF_1R blockade would attenuate increased ethanol drinking promoted by DID procedures.

METHODS

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were 6 to 8 weeks old, weighed between 25 to 30 g at the onset of each experiment, and were single-housed in polypropylene cages with corncob bedding. Standard rodent chow (Teklad, Madison, WI) and water were available at all times, except where noted. The vivarium rooms were maintained at an ambient temperature of 22°C with a 12 h/12 h light–dark cycle. Lights came on at 10:30 PM and went off at 10:30 AM. All experimental procedures were approved by the University of North Carolina Animal Care and Use Committee (IACUC) and were in compliance with the NIH Guide for Care and Use of Laboratory Animals.

Drugs

CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) was donated by Pfizer (Groton, CT), and was suspended in a vehicle of 0.5% carboxymethylcellulose (CMC). CP-154,526 displays high affinity for the CRF_1R (K_i < 10 nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al., 1996; Schulz et al., 1996). Importantly, peripheral administration of CP-154,526 has been shown to cross the blood-brain barrier and reach peak brain concentrations 20 minutes after administration with significant levels of the drug observed in the cortex, striatum, cerebellum, and hippocampus (Keller et al., 2002). Additionally, intraperitoneal (i.p.) injection of CP-154,526 in the dose range examined here appears to produce antidepressant-like and anxiolytic-like effects in rodents (Breese et al., 2004; Chen et al., 1997; Lundkvist et al., 1996; Mansbach et al., 1997), data that suggest functional central actions of this drug when it is administered peripherally. All concentrations of CP-154,526 used in the present experiment were mixed such that the final injection volume was 5 ml/kg. To habituate mice to procedures, all mice handled were given i.p. injections of CMC (5 ml/kg) daily for approximately 7 days before the initiation of the experiments. The site of injection was switched daily in an attempt to limit discomfort and tissue damage.

Experiment 1: DID After Administration of CP-154,526 With 4-Hour Training Sessions

All mice (n = 39) underwent a modified DID protocol (Rhodes et al., 2005). Briefly, all homecage water bottles were replaced with a single bottle of 20% (v/v) ethanol, 3 hours into the start of the dark phase. The 20% ethanol solution remained on the homecage for 4 hours. All mice had ad libitum access to food during this time. After the 4 hour session, the 20% ethanol bottle was replaced with a bottle containing water. On the first 3 days of this procedure, mice were given an i.p. injection of CMC, 30 minutes prior to the presentation of the ethanol bottle. Mice were then distributed into 4 groups, matched for an average ethanol consumption that occurred over the first 3 days of the experiment (that is, the mice were distributed so that the baseline level of ethanol consumption was approximately equal between the groups). On the fourth day, mice were given an i.p. injection of one of 4 doses of CP-154,526 (0, 1, 3, 10 mg/kg) mixed in CMC, 30 minutes prior to the application of the ethanol bottle. Immediately following the 4 hour test session, tail blood (6 μl) was collected from mice to determine BECs.

Experiment 2: DID After Administration of CP-154,526 With 2-Hour Training Sessions

Procedures for this experiment were similar to those used in Experiment 1 except that mice (n = 40) had access to the ethanol bottle for 2 hours (rather than 4 hours) during days 1 to 3. As above, mice were given an i.p. injection of CMC, 30 minutes before access to ethanol, and mice were distributed to 4 groups matched for average ethanol consumption that occurred over the first 3 days of testing. On the fourth day, mice were injected with one of 4 doses of CP-154,526 (0, 1, 3, 10 mg/kg) mixed in CMC, 30 minutes prior to the application of the ethanol bottle. Immediately following the 4 hour test session, tail blood (6 μl) was collected from mice to determine BECs. This alternate DID procedure was used because Rhodes et al. (2005) found that shortening the length of ethanol access during the first 3 days of training led to greater ethanol consumption and greater BECs on the fourth day of access.

Experiment 3: Open-Field Locomotor Activity After Administration of CP-154,526

To determine whether CP-154,526 could impair locomotor activity, naïve male C57BL/6J mice (n = 20) were tested in an open-field arena that automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA). The open field arena

measured 40.64 × 40.64 × 30.48 cm and was made of clear Plexiglass. Several cms of corncob bedding were placed into the open field chamber to aid in cleaning and to prevent the buildup of odor. C57BL/6J mice were handled and injected with CMC daily for 7 days before activity testing. CMC or CP-154,526 (10 mg/kg) was administered to mice ($n = 10$ per group) and then 30 minutes later, mice were placed in the center of the locomotor activity chamber. All mice were tested beginning 3 hours into the dark cycle to match DID procedures. Horizontal distance traveled (in centimeters) was recorded as an index of motor function during the 4-hour test session.

Experiment 4: Sucrose DID After Administration of CP-154,526 With 2-Hour Training Sessions

To determine if a 10 mg/kg dose of CP-154,526 had a general suppressive effect on consummatory behavior, male C57BL/6J mice ($n = 20$) were tested with procedures similar to those used in Experiment 2 except that the solution used for each 2-hour training session and the 4-hour test session was a 10% (w/v) sucrose solution. Mice were habituated to i.p. injections with CMC over 7 days and were also given i.p. injections of CMC on days 1 to 3. Mice were distributed to 2 groups matched for average sucrose consumption that occurred over the first 3 days, and were injected with CMC or a 10 mg/kg dose of CP-154,526 ($n = 10$ per group), 30-minutes prior to the 4-hour test on day 4.

Blood Ethanol Concentrations After Administration of CP-154,526

Blood ethanol samples were analyzed with gas chromatographic methods described elsewhere (Knapp et al., 1993; Navarro et al., 2003). Tail blood (6 μl) and standards (6 μl; 0 to 300 mg/100 ml) were mixed with 375 μl of distilled water and 0.5 g of NaCl in 12 × 75 mm borosilicate glass culture tubes. The tubes were capped and then heated at 55°C for 10 minutes in a water bath, at which point 1.5 ml of headspace gas was removed with a plastic 3.0 ml syringe and injected directly into an SRI 8610C gas chromatograph (SRI Instruments, Torrance, CA) equipped with an external syringe adapter and a 1.0 ml external loading loop. The oven temperature was isothermal at 140°C and contained a Hayesep D column and a flame ionization detector. Hydrogen gas, carrier gas (also hydrogen), and internal air generator flow rates were 13.3, 25, and 250 ml/min, respectively. Peak retention time was 2 minutes, and the areas under the curve were analyzed with SRI PeakSimple software (SRI Instruments) for Windows running on a Dell (Dell, Round Rock, TX) Inspiron 3500 laptop computer.

Data Analysis

All data in this report are presented as means ± SEM. One-way analyses of variance (ANOVA) were used to analyze data from Experiments 1 and 2. When significant main effects were obtained, Tukey's HSD post hoc tests were performed for group comparisons (Winer et al., 1991). For Experiment 3, a repeated measures ANOVA was used to analyze locomotor activity data over the 4-hour session. For Experiment 4, an independent student's t-test was performed to assess sucrose consumption data. Significance was accepted at $p < 0.05$ (two-tailed).

RESULTS

Experiment 1: DID After Administration of CP-154,526 With 4-Hour Training Sessions

The volume of ethanol consumed (g/kg) and BECs achieved following 4 hours of access to ethanol on day 4 of Experiment 1 are presented in Fig. 1*A* and 1*B*, respectively.

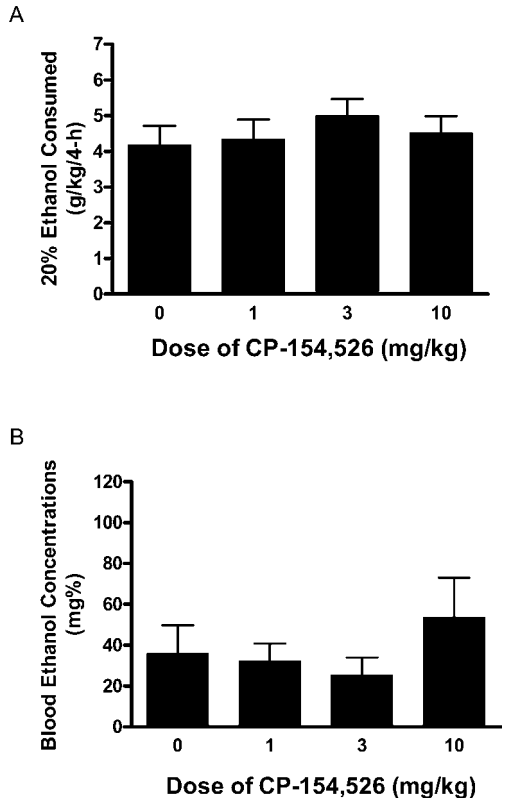

Fig. 1. Consumption of 20% (v/v) ethanol (**A**) and blood ethanol concentrations (BECs) (**B**) following the 4-hour ethanol consumption test on day 4 of Experiment 1. Mice were given an intraperitoneal (i.p.) injection of the CRF_1R antagonist CP-154,526 (0, 1, 3, 10 mg/kg) 30 minutes before access to ethanol. There were no significant differences between treatment groups. All values are means ± SEM.

Mice pretreated with the 0, 1, 3, and 10 mg/kg doses of CP-154, 526 drank 26.13 ± 3.35, 27.11 ± 3.47, 31.31 ± 2.85, and 28.21 ± 2.92 ml/kg/4 h of ethanol, respectively. One-way ANOVAs performed on these data revealed no significant effects of pre-treatment with CP-154,526 on the amount of ethanol consumed [$F(3, 35) = 0.504$, $p = 0.682$] or BECs [$F(3, 35) = 0.829$, $p = 0.487$].

Experiment 2: DID After Administration of CP-154,526 With 2-Hour Training Sessions

The volume of ethanol consumed (g/kg) and BECs achieved following 4 hours of access to ethanol on day 4 of Experiment 2 are presented in Fig. 2*A* and 2*B*, respectively. Mice pretreated with the 0, 1, 3, and 10 mg/kg doses of CP-154, 526 drank 37.89 ± 3.23, 40.03 ± 5.14, 32.79 ± 4.73, and 17.81 ± 4.64 ml/kg/4 h of ethanol, respectively. A one-way ANOVA performed on ethanol consumption data was significant [$F(3, 36) = 4.961$, $p = 0.006$]. Tukey's HSD post hoc tests revealed that the 10 mg/kg dose of CP-154,526 significantly reduced ethanol consumption relative to the control group. Neither 1 nor 3 mg/kg doses of CP-154,526 significantly altered ethanol consumption relative to the CMC-treated group. A one-way ANOVA performed on BEC data was

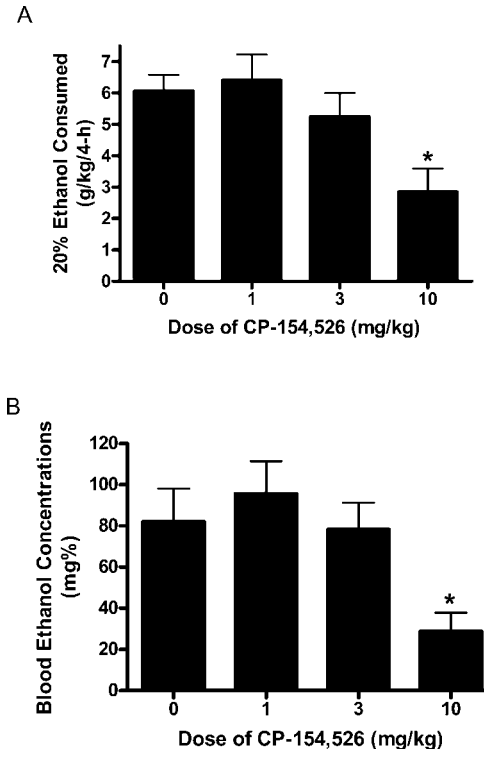

Fig. 2. Consumption of 20% (v/v) ethanol (**A**) and blood ethanol concentrations (BECs) (**B**) following the 4-hour ethanol consumption test on day 4 of Experiment 2. Mice were given an intraperitoneal (i.p.) injection of CP-154,526 (0, 1, 3, 10 mg/kg) 30 minutes before access to ethanol. Relative to mice treated with carboxymethylcellulose (CMC), pretreatment with the 10 mg/kg dose of CP-154,526 caused a significant reduction of ethanol consumption and BECs. All values are means ± SEM. *$p < 0.05$ relative to the CMC treatment group.

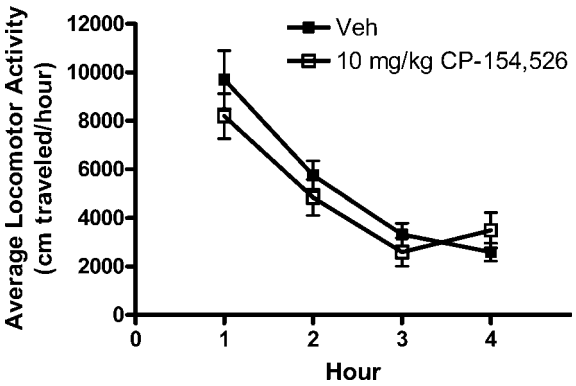

Fig. 3. Open-field locomotor activity (cm/h) during the 4-hour test following intraperitoneal (i.p.) injection of CP-154,526 (10 mg/kg) or the vehicle (Veh) 30 minutes before testing. There were no significant differences between the drug pretreatment groups at any time point. All values are means ± SEM.

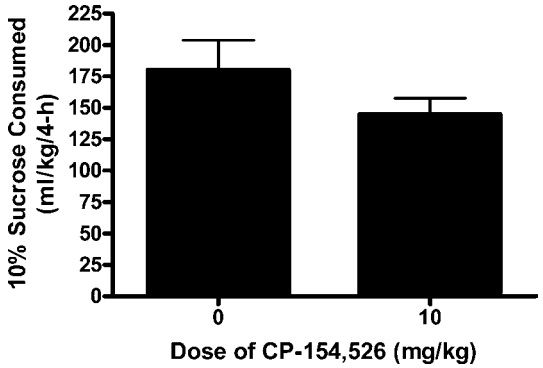

Fig. 4. Consumption of a 10% (w/v) sucrose solution following the 4-hour sucrose consumption test on day 4 of Experiment 4. Mice were given intraperitoneal (i.p.) injection of CP-154,526 (0, 10 mg/kg), 30 minutes before access to sucrose. There was no significant difference between the two groups. All values are means ± SEM.

significant [$F(3, 36) = 4.493$, $p = 0.009$], and Tukey's HSD post hoc tests showed that the group treated with the 10 mg/kg dose of CP-154,526 displayed significantly lower BECs relative to the CMC-treated group. Groups pretreated with the 1 or 3 mg/kg doses of CP-154,526 did not display BECs that were significantly different from the CMC treated group.

Experiment 3: Open-Field Locomotor Activity After Administration of CP-154,526

Data representing 4 hour locomotor activity following i.p. injection of CMC or a 10 mg/kg dose of CP-154,526 are presented in Fig. 3. A 2 × 4 (dose × hours) repeated measures ANOVA run on the locomotor activity data revealed a main effect of hour [$F(3, 54) = 67.614$, $p = 0.001$]. However, neither pretreatment with CP-154,526 (dose) nor the interaction between dose and hours were statistically significant.

Experiment 4: Sucrose DID After Administration of CP-154,526 With 2-Hour Training Sessions

The volume of sucrose consumed (ml/kg) following 4 hours of access to ethanol on day 4 of Experiment 1 are presented in Fig. 4. An independent t-test performed on these data did not achieve statistical significance [$t(18) = 1.330$, $p = 0.205$].

DISCUSSION

Here, we demonstrate that i.p. injection of a 10 mg/kg dose of CP-154,526, a selective CRF_1R antagonist, significantly attenuated ethanol consumption and BECs in C57BL/6J mice when DID procedures that promoted high levels of ethanol consumption (approximately 6.0 g/kg/4 h) were employed (Experiment 2). Interestingly, CP-154,526 had no effect on ethanol consumption or BECs, when DID procedures that promoted more moderate levels ethanol consumption (approximately 4.0 g/kg/4 h) were employed (Experiment 1). These observations suggest high, but not moderate levels of ethanol consumption induced by specific DID procedures are modulated by CRF_1R signaling.

It was possible that the 10 mg/kg dose of CP-154,526 reduced ethanol consumption because of non-specific effects,

such as impairment of motor function or general reductions of consummatory behavior. To determine the effects of CP-154,526 on motor function, a control experiment (Experiment 3) was performed to assess the effects of the 10 mg/kg dose of CP-154,526 on locomotor activity over a 4-hour test 3 hours into the dark cycle. The 10 mg/kg dose of CP-154,526 did not significantly alter 4-hour open-field locomotor activity, thus the ability of this dose of CP-154,526 to reduce ethanol drinking in Experiment 2 was probably not related to effects of this drug on motor function. Importantly, pre-treatment with the 10 mg/kg dose of CP-154,526 did not influence high level of consumption of 10% sucrose over 4 hours when access began 3 hours into the dark cycle (Experiment 4), and this dose did not alter moderate ethanol consumption in Experiment 1. Both observations suggest that reduced ethanol drinking induced by pretreatment with CP-154,526 in Experiment 2 is probably not related to nonspecific effects of this drug on consummatory behavior. Rather, it appears that CP-154,526 specifically modulates ethanol drinking when consumption levels are elevated.

These data present novel evidence suggesting that CRF_1R signaling is involved with modulating high or excessive binge-like ethanol consumption in C57BL/6J mice that are induced by specific DID procedures. Interestingly, these observations parallel previous data, where antagonism of CRF receptors attenuated increased ethanol drinking in rodents made dependent to ethanol by exposure to ethanol diet or ethanol vapor, but had no effect on moderate levels of ethanol consumption in nondependent rodents (Finn et al., 2007; Sabino et al., 2006; Valdez et al., 2002). While ethanol drinking associated with DID procedures is unlikely to promote ethanol dependence to the degree achieved by exposure to ethanol vapor or ethanol-containing diets, the present findings, in tandem with previous work, suggest that CRF_1R signaling modulates increased ethanol drinking induced by a variety of rodent models. Ethanol exposure induces rapid activation (within 15 minutes) of HPA-axis signaling (Rivier, 1996; Rivier et al., 1990), an effect which is attenuated by pretreatment with CRF receptor antagonists (Rivier and Lee, 1996). Because CP-154,526 has been shown to attenuate stress-induced activation of HPA-axis activity (Arborelius et al., 2000; Xu et al., 2005), it is tempting to speculate that increased ethanol drinking associated with DID procedures is mediated, in part, by an up-regulation of HPA-axis activity, an effect which may be prevented by pretreatment with the CRF_1R antagonist. Consistently, treatment with corticosterone (a hormone that is secreted with HPA-axis activation) increases daily ethanol drinking by rodents, while inhibition of endogenous corticosterone synthesis or adrenalectomy suppress ethanol consumption (Fahlke et al., 1994, 1995, 1996). However, it should be noted that corticosterone pretreatment blocked the acquisition ethanol-induced conditioned place preference (CPP) in the TO strain of mice (Brooks et al., 2004), while a corticosterone synthesis inhibitor did not alter the expression or acquisition of CPP in DBA/2J mice (Chester and Cunningham, 1998), suggesting that corticosterone may not modulate ethanol's reinforcing properties. The possible role of HPA-axis activity in the modulation of increased ethanol drinking with DID procedures, or extrahypothalamic CRF signaling if involved, will be the topic of future research.

Consistent with Rhodes et al. (2005), we show here that the level of ethanol consumption is sensitive to the specific DID procedures. Thus, the highest levels of ethanol consumption occurred when mice had 2 hours of access to ethanol during the first 3 days of the procedure and 4 hours of ethanol access on the final test day when BECs were assessed (Experiment 2). With this procedure, mice achieved BECs of approximately 80 mg%. On the other hand, when mice had access to ethanol solution for 4 hours on each of the 4 days of the experiment, mice achieved BECs of approximately 30 mg% (Experiment 1). However, despite higher levels of ethanol consumption with procedures used in Experiment 2, the level of ethanol consumption and the associated BECs were lower than those reported by Rhodes et al. (2005) using identical procedures and the same strain of mice. It is likely that subtle environmental differences between laboratories are the bases of differences in the level of ethanol consumption between the present observations and those previously reported (Rhodes et al., 2005), as environmental factors have been demonstrated to have significant impact on behavioral measures (Crabbe et al., 1999; Wahlsten et al., 2003).

In conclusion, this study demonstrates that i.p. administration of the systemically bioavailable and selective CRF_1R antagonist, CP-154,526, reduces excessive ethanol consumption caused by specific DID procedures. These results are consistent with research showing that the CRF system modulates a spectrum of neurobiological responses to ethanol. A recent report found that both naltrexone and the dopamine re-uptake inhibitor GBR 12909 can attenuate increased ethanol consumption associated with DID procedures, suggesting a role for opioid and dopamine receptor signaling (Kamdar et al., 2007). The present observations add to this small but growing literature by demonstrating that CRF_1R signaling selectively modulates high ethanol drinking without altering moderate levels of ethanol consumption or sucrose drinking. Future research is needed to determine the brain regions in which CRF_1R signaling modulates increased ethanol drinking associated with DID procedures and if the CRF_2 receptor plays a role.

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